RECOMBINATION BETWEEN THE INFLUENZA VIRUS STRAINS WSE AND CAM

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For some years our work on recombination between influenza viruses has been largely concerned with derivatives of the two classical strains WS and MEL (Melbourne, 1935). Work with these has led to the recognition of three types of genetic interaction.

(1) The appearance of doubly neutralizable virus in part to be regarded as phenotypic mixtures (mostly non-viable), in part as true heterozygotes (Fraser, 1953; Burnet and Lind, 1954a; Hirst, 1953; Gotlieb and Hirst, 1954).

(2) Interchange of linkage groups of the type that can be represented A-C+a-c⇌A-c+a-C. In our experiments the strains MEL+WSE⇌M+, +WS— were used (Burnet and Lind, 1952).

(3) Redistribution of virulence between strains of widely different virulence, e.g., the neuropathogenicity of NWS could be transferred to MEL and the capacity to produce haemorrhagic lesions in the chick embryo, on transfer from WSE to MEL, showed a wide range of activity (Burnet and Lind, 1954b).

Other strains such as the B strains LEE and MIL (Melbourne, 1945) and various derivatives of CAM (A prime Melbourne, 1946) have also been used to study specific points (Perry, van den Ende and Burnet, 1954; Burnet and Lind, 1954d). The present work was designed primarily to see whether the genetic behaviour of the much more recent A prime strains CAM would fit into the pattern of linkage groups found for MEL and WS variants.

In the course of the work a number of other aspects of interest emerged. The main conclusion was that, although the yield of recombinants between WSE and CAM was much smaller, the types obtained were generally analogous to the M+ and WS— recombinants of the earlier experiments. An interesting new feature was the indication that for recombination with CAM to take place the genetic structure of WSE must provide a different type of linkage group from that involved in recombination between WSE and MEL.

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MATERIALS AND METHODS.

Virus strains.

The following two influenza strains were used:

WSE—a substrain of the classical WS influenza type A strain (Andrewes, Laidlaw and Smith, 1934) which was derived by adaptation to growth on the chorioallantois and which has been used in many other investigations.

CAM—an influenza A prime strain (Anderson and Burnet, 1947). The virus used in these experiments was isolated in the chick embryo and had undergone 7 amniotic and 8 allantoic passages.

Freshly harvested virus from embryos inoculated two days previously with seed virus at a dilution of 10^{-4} was used for experimental work.

Preparation of anti-sera.

Anti-serum to the WSE strain was prepared by intravenous inoculation of 5 ml. of undiluted WSE allantoic fluid into a rabbit which was bled out 10 days later.

CAM anti-serum was produced by intranasal inoculation of a ferret with freshly harvested CAM allantoic fluid. The ferret was bled out 10 days after infection.

The sera were inactivated by heating at 56° C. for 30 minutes. Portion of each serum was tubed in small amounts and stored in the frozen state (−20° C.) and subsequently used in all *in vivo* work. The remaining serum was treated with twice its volume of M/100 potassium periodate for 30 minutes at room temperature, after which glycerol was added to give a final serum dilution of 1/5 (Burnet and Lind, 1954c). This was stored at 4° C. and used in all *in vitro* work. The sera were highly specific when tested in the allantoic cavity. The ID_{50} titres obtained when virus dilutions were made in anti-serum (diluted 1/25) instead of in normal horse serum saline showed the following reductions:

CAM in anti-CAM $10^{-6.3}$ in anti-WS $10^{-1.0}$.

WSE in anti-WSE$>10^{-7.3}$ in anti-CAM$^{-0.7}$.

Anti-haemagglutinin titrations with periodate-treated serum gave titres of between 1000 and 2000 against homologous virus and less than 100 against heterologous virus.

Diluents.

Saline: 9 gm. of NaCl made up to 1000 ml. with distilled water.

Normal horse serum saline (NHSS): Saline containing 10 p.c. inactivated normal horse serum.

Glucose Ringer containing antibiotics (GPSR): Ringer's solution (NaCl 0·85 gm., KCl 0·025 gm. and $CaCl_2$ 0·03 gm. made up to 100 ml. with distilled water) to which glucose (0·1 p.c.), penicillin (20 units per ml.) and streptomycin (70 mμ per ml.) are added.

Titration of haemagglutinin (HA).

Doubling dilutions of virus fluid were prepared in 0·25 ml. volumes of saline and an equal volume of a 1 p.c. suspension (V/V) of fowl red blood cells was added to each tube. After standing for 45 minutes at room temperature, the end point was determined from the pattern of the agglutinated cells and the reciprocal of the dilution giving the standard "+" reaction of partial agglutination was taken as the titre. Where necessary this was determined by interpolation.

For many purposes fluids were diluted to a standard level in terms of haemagglutinin. In all cases the HA titre was regarded as measuring a concentration, not an absolute amount. If, for example, a fluid gave a HA titre of 500 by the above method, it would be diluted 1/500 and 0·05 ml. of this dilution inoculated intranasally into each mouse. In the text this is referred to as testing mouse pathogenicity at 1 agglutinating dose (1 AD). Similarly, if a 5 ml. amount of this dilution is used in a de-embryonated egg it will also be referred to as virus 1 AD.

Recombination experiments.

These followed the pattern previously described (Burnet and Lind, 1954a). Freshly harvested virus fluids of 40–44 hours' incubation were diluted to a level of 20 ADs in GPSR. The required mixtures were prepared and as a rule 2 de-embryonated eggs (DEE), prepared from chick embryos which had had 14–16 days' preliminary incubation, received 5 ml. of each mixture and were incubated at 35° C. on a rotating disc, as described earlier. The inoculum was removed after one hour and the DEE thoroughly washed with three lots of Ringer's solution before being recharged with 5 ml. of GPSR. No RDE was used in these experiments. The fluid was harvested 7 hours after the first inoculation and distributed into a number of ampoules for storage in a dry ice refrigerator. The DEE were usually refilled and a second harvest made at 22 hours, but in only one of the reported experiments was any use made of these fluids.

The first growth cycle fluids were titrated by the allantoic method at 10-fold dilutions or, in retitrations, at 0·5 $\log_{10}$ intervals near the end point and fluids from the highest positive dilution (LD) taken for testing. In most cases, the first cycle fluids were also tested by titration in anti-CAM and anti-WSE sera. All clones of virus were grown at least once in the presence of heterologous anti-serum to ensure that only the one serological type of virus was present, and in many cases the absence of heterologous virus was checked by passage in homologous immune serum.

Determination of double neutralization.

First cycle fluids from de-embryonated egg experiments were titrated for haemagglutinin titre, using as diluents saline and a 1/200 dilution of periodate-treated antiserum against each of the virus strains used (Burnet and Lind, 1954c). The sum of the titres obtained in the two antisera was expressed as a percentage of the titre in saline. It was found that with the controls, or with artificial mixtures of the two viruses concerned, the sum of the titres in the two antisera was 50 p.c. or more of the saline titres, so that a percentage of less than 50 p.c. was indicative of neutralization by both antisera.

Characterization of virus clones.

The methods used for characterizing virus fluids were essentially those used in earlier work (Burnet and Lind, 1952). The only important modification was in regard to the heat treatment of fluids before testing for indicator status. We found that when CAM strains were heated at 56° C. the results of such tests were highly variable, depending mainly on the pH of the fluid. Dr. J. F. O'Dea kindly carried out a limited study of the position and found that completely regular results could be obtained if to one volume of allantoic fluid an equal volume of veronal buffer (0·06 M), pH 7·0 (Vogel, 1944) was added and the fluid then heated at 52° C. for 30 minutes. This technique was equally effective in differentiating WSE and WS– strains and was used as a standard in all tests.

The marker characteristics used were essentially the same as those used in work on MEL/WSE recombination (Burnet and Lind, 1952), and brief notes only will be given in regard to the differentiating features and the technique used to demonstrate them.

A'a. Serological character as tested by haemagglutinin inhibition with periodate-treated antisera. The antigenic character of CAM represented by A' is quite distinct from that of WSE (a) and in many tests single antiserum dilutions of 1:200 were used against 5AD of virus. If results were at all equivocal a full titration, using falling dilutions of each antiserum, was carried out.

b. The heat resistance of CAM is relatively low and not significantly different from that of WSE. The character was not tested for as a routine and, where b is inserted in the formula of various combinants, this is merely to allow them to be compared more readily with the types obtained in earlier MEL/WSE recombination experiments.

Cc. Conversion to indicator. Under the conditions described above—heating at 52° C. in veronal buffer at pH 7·0—there is a sharp difference between CAM which is not converted to indicator status (C) and WSE which is (c). The recombinants show equally consistent behaviour CAM+ being converted to indicator and WS− failing to change under these conditions. The inhibitor used was semipurified meconium inhibitor (Curtain, French and Pye, 1953), tested in 10-fold dilutions against 5AD of heated virus, the red cells being added after 30 minutes' contact at room temperature between the reagents.

Dd. Indicator status of heated virus for sheep salivary gland mucoid. These tests were done in parallel with the preceding ones. WSE and, to a slightly diminished extent, WS− are converted to indicator status (d) while all CAM derivatives are unaffected under these conditions (D).

Ee. Pathogenicity for chick embryo. The strain WSE is highly pathogenic, killing at 3 days or less and producing extensive haemorrhagic lesions (e). CAM has no pathogenic action (E). For this test, freshly harvested fluids were diluted to a titre of 1 AD in NHSS and 0·05 ml. of this dilution inoculated on the chorioallantois of 4, 12-day chick embryos. After 3 days' incubation at 35° C. the eggs were candled and dead embryos examined for lesions; all the remaining embryos were examined on the fourth day. Details recorded were time of death and the intensity of haemorrhagic lesions in brain muscles and lungs. In the Tables, the average intensity of haemorrhagic lesions is indicated by grading from +++ to −.

Ff. Pathogenicity for mouse lung. WSE is highly pathogenic, 0·1 AD of virus giving complete or nearly complete consolidation (f); CAM has no trace of activity at 1 AD (F). All tests are made by diluting the virus fluid to 1 AD if it is serologically CAM, to 0·1 AD if it is serologically WS, and inoculating 4 mice under chloroform-ether anaesthesia with 0·05 ml.

The mice were killed 7 days after inoculation and the lung lesions were scored 1–3·9 for living mice according to the degree of consolidation. Death on the 5th day or earlier scored 5, on the 6th day 4·5 and on the 7th day 4. If the average score of lung lesions from a 1 AD inoculum is less than 0·1, the strain is graded F, anything higher as f. It should be noted that in the MEL/WSE series, F was used when 1 AD failed to kill mice within 7 days, most MEL strains giving lesion scores between 1 and 3.

The composition of the strain CAM can then be represented as A'bCDEF in comparison with MEL ABCDEF and WSE abcdef. In the first instance experimentally-obtained strains were characterized by serological character and indicator state as tested with meconium inhibitor. This allowed differentiation into the original forms CAM and WSE and the recombinants CAM+ and WS−. CAM+ in this respect is analogous to the M+ of previous studies.

EXPERIMENTAL.

Interaction between CAM and WSE.

All the results to be reported in this section were from the analysis of first cycle fluids obtained from a single experiment in which pairs of de-embryonated eggs received CAM alone, WSE alone and mixtures in the ratio 5:1, 1:1 and 1:5 respectively. Each fluid was stored in several ampoules in the CO_2 ice box and retitrated under various conditions to obtain limit dilution fluids which were then studied for the five qualities A'a serological type; Cc and Dd, indicator status when tested against meconium and sheep salivary mucoid inhibitors; Ee

capacity to produce haemorrhagic lesions in chick embryos inoculated on the chorioallantois and Ff the power to produce lung consolidation in mice. As will emerge in the discussion, the existence of heterozygosis makes it inadvisable to rely on simple limit dilution titration to provide a uniform clone of virus, and all those clones whose characters are reported had at least one passage in the presence of heterologous immune serum to ensure that they were at least serologically homozygous. Clones used for detailed study had several re-isolations from eggs inoculated with limit infective dilutions and were shown to be serologically homogeneous by allantoic inoculation with both homologous and heterologous antisera.

TABLE 1.

In vitro characters of first cycle fluids of CAM/WSE experiment.

Viruses used to inoculate DEE	Haemagglutinin titre of 7 hour fluid in			Double neutralization
	Saline	AntiCAM/200	AntiWSE/200	*
CAM alone C	80	<2	40	−(50%)
WSE alone W	120	60	<2	−(50%)
5 CAM+1 WSE C5/W1	110	3	5	++(7·5%)
1 CAM+1 WSE C1/W1	160	7	2	++(5·5%)
1 CAM+5 WSE C1/W5	200	35	<2	+(18%)

* Sum of the HA titres in the two antisera expressed as a percentage of the HA titre in saline.

The results can be expressed in a series of Tables. Table 1 shows the qualities of the first cycle fluids from which it will be seen that all the fluids from double infections show some degree of double neutralization. In the C1/W5 fluid there is clearly a great excess of WS type virus.

Allantoic titrations showed, as would be expected from earlier experience (Burnet, Lind and Stevens, 1955), that CAM alone gave nearly the expected ID_{50} while WSE alone was highly incomplete, the ID_{50} $10^{-4.5}$ being only 1/1000 of that to be expected from the haemagglutinin titre. The C1/W1 mixture with an overall ID_{50} of $10^{-6.3}$ was intermediate in character and again the WS content appeared to be more incomplete than the CAM content.

Detailed titrations of the C1/W1 first cycle fluid were made by allantoic inoculation, dilutions being prepared in normal serum saline and in dilute anti-serum—anti-CAM or anti-WS diluted in each case 1:100. This was chosen as the highest concentration of antiserum with no significant action on the titre of the heterologous virus. The results of several titrations are combined in Table 2.

It will be seen that there appears to be a significantly greater reduction of the titre of the heterologous component than would be expected from the absence

TABLE 2.

Titration of infectivity of first cycle fluid C1/W1 in three diluents.

Dilution	Serological type of positive fluids obtained when dilutions made in					
	Normal Serum Saline		AntiCAM Serum		AntiWSE Serum	
	CAM	WS	CAM	WS	CAM	WS
10^{-7}	1	1 (15)				
$10^{-6.5}$	5	4 (15)				
10^{-6}	12	5 (28)	0	2 (14)	3	0 (14)
10^{-5}			2	6 (15)	11	0 (16)
Equivalent ID_{50}	$10^{-6.1}$	$<10^{-6.0}$		$10^{-5.0}$	$10^{-5.4}$	

Figures in brackets represent number of eggs inoculated with each dilution.

of action on normal heterologous virus but, in view of the partially incomplete character of the virus used, no special stress is laid on this point.

In view of the likelihood that inoculation of the unbalanced mixtures would yield an excess of unaltered virus of one type, titrations of the first cycle fluids C5W1 and C1W5 were made only in the presence of the antiserum corresponding to the major component.

The fluid C5W1 in anti-CAM (1:25) gave an ID_{50} of $10^{-4.5}$ while C1/W5 in anti-WSE (1:25) gave a value of $10^{-4.1}$. Limit dilution fluids from all these titrations were tested for the qualities mentioned above, those obtained from titrations in normal serum saline being passed once more in the heterologous serum before testing. The characteristics of these presumptive pure clone fluids obtained directly from first cycle fluids are summarized in Table 3.

TABLE 3.

Character of clones obtained from CAM/WSE interaction.

Serological and indicator status	Mouse pathogenicity		Embryo haemorrhages	No. of clones
	Dose	Lung Lesions		
WSE	0·1AD	3·5+	All+++	7
		2·0–3·4	7+++1–	8
		0·2–1·9	3–	3
		0–0·1	2+++1–	3
WS–	0·1AD	3·5+	2–	2
		2·0–3·4	1–	1
CAM	1·0AD	0·2+	2–	2
		0–0·1	18–	18

For comparison, 7 LD fluids were obtained from a titration of the first cycle fluid from WSE alone. These were all of WSE type *in vitro* and in mice gave values for 0·1 AD inocula of 3·4 or higher except for one clone with a value of 1·6.

It will be seen first that CAM strains of normal character only were isolated except for 2 with minimal but definite capacity to produce lung lesions. Fourteen essentially normal strains of WSE were isolated although a much wider range of mouse pathogenicity was encountered than would be found in standard allantoic passage material. Seven strains of WSE with greatly diminished mouse and/or embryo pathogenicity were obtained, and 2 WS— strains, two of standard type and one with somewhat low mouse pathogenicity. No significant differences were visible between the types of strains from the 3 first cycle fluids, and they were not differentiated in the Table.

The absence of any evidence of CAM type recombinants analogous to M+ strains of earlier experiments (Burnet and Lind, 1952) was disappointing, so further tests were made in which a highly selective environment was provided.

TABLE 4.

Titration of fluid C1/W5 on choriollantois with Anti WS serum 1 : 10.

Dilution	Number positive	Characteristics of individual embryos		
		Serial no.	Lesions	Virus isolated
10^{-1}	3/18	49863	+++	CAM+
		50467	+++	WSE only
		50475	++	CAM+, WSE
10^{0}	6/10	50478	−	CAM, WS−
		50485	+++	CAM+

TABLE 5.

Characterization of CAM+ and WS− strains from chorioallantoic experiment.

Strain & LD descendants	Type	Mouse pathogenicity		Embyro lesions
49863	CAM+	1·0AD	1·3	+++
1st LD 50136	CAM+		2·4	+++
2nd LD 50809	CAM+		2·2	+++
3rd LD 50878	CAM+		1·5	+++
50475	Mixture			
1st LD 50781	CAM+		1·3	+++
2nd LD 50782	CAM+		1·3	+++
50485	CAM+			
1st LD 50797	CAM+		1·8	+++
2nd LD 50798	CAM+		0·3	+++
50478	Mixture			
1st LD 50718	WS−	0·1AD	0·9	−
2nd LD 50721	WS−		0·0	−

If recombinants analogous to M+ were to be obtained, they should produce haemorrhagic lesions in the chick embryo after inoculation on the chorioallantois with an excess of anti-WSE serum. This manoeuvre proved successful with the C1/W5 first cycle fluid and what we call CAM+ strains were isolated from fluid mixed undiluted, or at 1 : 10, with a 1 : 10 dilution of anti-WS serum. There was a large excess of WSE in the fluid and some of the embryos showing haemorrhagic lesions contained WS type virus. The general technique was to harvest the lungs of all embryos dead by the 4th day after inoculation. Emulsions of these lungs usually contained enough haemagglutinin to allow a presumptive test of serological character, and those which appeared to contain CAM were titrated in the allantoic cavity with anti-WSE serum to isolate the recombinant. In most cases, any WS strain was also isolated and characterized.

The CAM+ strains are clearly uniform in character with very active pathogenicity for the chick embryo and a moderate capacity to produce mouse lung consolidation. The strain 49863 had 5 consecutive passages at limit dilution and retained all its character at each passage.

The WS— strain present, together with CAM in lung 50478, was similarly carried for five LD passages, retaining throughout its lack of pathogenicity for both chick embryos and mice.

In several instances allantoic fluids from eggs inoculated with the dilution below limit infecting dilution in titrations of first cycle fluids were examined by titration in both antisera, and in most cases both serological types of virus were isolated. In the development of these fluids many opportunities for secondary recombination processes must have been presented and some clones with unusual characters were obtained.

Fluid 47815 with WS serological character gave typical WSE fluids in two LD titrations in which NHSS was used as diluent. Titrated in anti-WS serum, the two CAM strains obtained at LD (10^{-3}) differed sharply. Both were of indicator type and fluid number 51744 showed the pathogenicity for mice and chick embryos typical of the CAM+ strains isolated by chorioallantoic inoculation. The other fluid, 51743, had no pathogenicity for either mice or chick embryos. On further LD passage these characters were reproduced in all fluids examined, 3 from 51743, 2 from 51744. These strains can be formulated

A′D-cef (CAM+) and A′DFE-c

In addition to those shown in Table 5, WS— strains non pathogenic or very weakly pathogenic for mice were obtained from fluids 47821 and 48775, obtained by LD titration of C1/W1, and from 47835, obtained from C5/W1, while two WSE strains nonpathogenic for mice were also obtained from C5/W1.

In summary, the CAM/WSE experiments allowed the isolation of clones of the following types:

(a) In very small yield the reciprocal recombinants CAM+ (A′bD-cef) and WS— (abd-CEF).

(b) A CAM+ strain lacking pathogenicity for mice and chick embryos A′bDFE-c.

(c) A variety of WS strains with varying degrees of loss of pathogenicity. In addition to WSE of typical character, clones, which can be formulated abdf-CE, abdFce, abdFcE and abdfcE, were obtained.

The most convenient way of showing the range of mouse pathogenicity with WSE strains derived from double infection is to contrast the behaviour of clones derived on the one hand from WSE alone and from inoculum W5/C1 where most WS clones would be derived from cells infected with WS only, and on the other hand from inocula W1/C1 and W1/C5, in which most would have come from a cell also infected with CAM.

TABLE 6.

Percentage of WSE clones showing various grades of mouse pathogenicity.

DEE inoculum from which clones derived	Grade of mouse pathogenicity					No. of clones
	0-1	1·1-2	2·1-3	3·1-4	4	
WSE 5/CAM 1 or WSE	0	14	0	50	36	14
WSE 1/CAM 5 or WSE 1/CAM 1	24	10	21	34	10	29

It is clear that the first group contains more than 80 p.c. of standard WSE fluids, but in the second there are many almost avirulent clones with a wide range of intermediate values.

TABLE 7.

Characters of first cycle fluids in CAM+/WS− recombination experiment.

Viruses used to inoculate DEE	7 hour fluid			22 hour fluid		
	Saline	diluent		Saline	diluent	
		a CAM/200	a WSE/200		a CAM/200	a WSE/200
CAM+ alone	160	3	120	50	3	40
WS− alone	180	140	<2	200	120	3
5 C+/1 W−	140	32	8	320	50	7
1 C+/1 W−	120	100	3	240	50	3
1 C+/5 W−	240	240	3	320	140	3

Figures show haemagglutinin titres in the diluents shown.

Back recombination between CAM+ and WS—.

The two stock strains described above were each taken through five LD passages and showed no evidence of heterogeneity. Fresh fluids CAM+ 51065 and WS— 51071 were then used for a recombination experiment completely analogous to that carried out with CAM/WSE. The *in vitro* qualities of the first cycle fluids were as shown in Table 7.

It will be seen that double neutralization is only evident in the 5C+/1W— first cycle fluid and that all show a relatively great excess of WS virus.

In order to obtain the recombinants corresponding to CAM and WSE, titrations were made allantoically with anti-WS and on the chorioallantois with anti-CAM serum. In addition, direct titrations without antisera were also made. The results of titrations in the allantoic cavity are shown in Table 8.

TABLE 8.

Allantoic titration of first cycle fluids CAM+/WS—

First cycle fluid	Diluent	ID_{50} and presence of recombinants	Approximate proportion of recombinants
C+5/W—1	NHSS	WS— $10^{-7.0}$ CAM+ $10^{-6.8}$ WSE (1/8* 10^{-7})	WSE 1/3
7 hrs	a CAM	WS— $10^{-6.2}$ WSE (2/10 10^{-6})	
	a WSE	CAM+ $10^{-5.2}$ CAM (1/10 10^{-4})	CAM 1/30
C+1/W—1	NHSS	WS— $10^{-7.2}$ WSE (1/8 10^{-6})	WSE 1/50
22 hrs	a WSE	CAM+ $10^{-3.0}$ CAM (2/8 10^{-3})	CAM 1/3
C+1/W—5	NHSS	WS— $10^{-7.0}$	
22 hrs	a WSE	CAM $10^{-3.0}$ (3/6 10^{-3} with 1/6 CAM+)	CAM 3/4

Diluents NHSS normal horse serum saline.
a CAM antiCAM ferret serum diluted 1 : 25.
a WSE antiWSE rabbit serum diluted 1 : 25.

* Ratio of number of eggs positive for the virus shown to the total number inoculated with the particular dilution of the first cycle fluid.

This Table presents a striking contrast to Table 3 in the case with which recombinants have been obtained. Instead of the presence of only $1/10^4$ recombinants, the number is almost as high as was obtained in M+/WS— recombinations, and where WS— is in excess the bulk of the CAM strains obtained are of the original CAM type.

It is entirely in line with these results that all three fluids tested on the chorioallantois with anti-CAM serum gave a high yield of WSE, C+5/W—1 10^{-4}, C+1/W—1 $10^{-4.5}$, C+1/W—5 $10^{-3.3}$. It is of interest that harvests from the lungs of some of these embryos gave dominantly WS—. This depends on the fact that although WS— does not produce haemorrhagic lesions, it frequently invades the circulation and multiplies freely in the lung.

Representative recombinant strains from each series have been carried for one or more further LD passages with the results shown in Table 9.

TABLE 9.

Representative passage series of recombinants from CAM+/WS− experiments.

DEE inoculum	Successive LD passage fluids and type	Mouse pathogenicity. Lesion scores	Chick embryo lesions
C+5/W−1	51640 CAM		
	1st LD 51905 CAM	2·1	− − −
	2nd LD 52393 CAM	1·3	
	51820 WSE		
	1st LD 51971 WSE	4·3	+ + +
	2nd LD 52176 WSE	4·5	+ + +
C+1/W−1	51889 CAM		
	1st LD 52108 CAM		
	2nd LD 52184 CAM	0	− − −
	51879 WSE	2·1	+ + +
	1st LD 52086 WSE		+ + +
	2nd LD 52203 WSE	4·1	+ + +
C+1/W−5	52014 CAM		
	1st LD 52193 CAM	0	− − −
	2nd LD 52508 CAM	0	− − −

[1]. Mouse pathogenicity: All strains serologically WS tested at 0·1AD, CAM strains at 1·0AD.

In summary, it has been found that typical back recombinants can be obtained very readily from double infections with CAM+ and WS— obtained from the first series of experiments. The only anomalous forms obtained were the CAM strains from C+5/W—1 shown in Table 9 which had definite pathogenicity for mice, i.e., A′bDf-CE and a WSE strain of low and variable pathogenicity for mice, not shown in the Table.

Recombination between back-recombinant WSE and CAM.

It was thought of interest to see whether the back recombinant WSE (R-WSE) from the reaction A′bD-fce+abd-FCE→abd-fce would retain a power to interact easily with CAM, owing to the existence of the presumed 3-3 distribution of genetic factors. A recombinant WSE clone 51820 from C+5/W—1 (see Table 9) was titrated in anti-CAM serum and a limit dilution fluid given 3 more LD pasasges with retention of all WSE characters by 2 fluids tested at each passage. This 4th passage fluid was then used in a recombination experiment with stock CAM virus. The first cycle fluids showed double neutralization and in each case these were titrated on the chorioallantois in the presence of anti-WSE serum to determine the proportion of CAM+ strains obtainable. The results shown in Table 10 are virtually identical with those of Table 4. Clearly the R-WSE obtained by back recombination has acquired no modification toward a 3-3 distribution of characters in the process.

The only significant difference from the first experiment is that the CAM+ clones obtained have a lower mouse pathogenicity. Of those shown in Table 10,

7 have been tested giving lung lesion scores at 1AD of 0·5, 0·4, 0·2, 0·15, 0·1, 0·0, 0·0. All were typically pathogenic for the chick embryo. The results, therefore, indicate the appearance of a significant proportion of recombinants of AbDF-ce type which was not found in the initial experiment.

TABLE 10.

Clones obtained from interaction between CAM and back recombinant WSE (R—WSE).

Dilution	Recombinant clones obtained by chorioallantoic inoculation of 1st cycle fluids from DEE inoculated with the following CAM/R-WSE mixtures					
	CAM 5/R-WSE 1		CAM 1/R-WSE 1		CAM 1/R-WSE 5	
	Number positive	Virus present	Number positive	Virus present	Number positive	Virus present
10^{-2}	1/4	CAM+	0/4		0/4	
10^{-1}	3/4	CAM+	2/4	CAM+	0/4	
10^{0}					1/4	CAM+

DISCUSSION.

There are four aspects of the present experiments which may be discussed separately in the first instance.

(1) Recombination by "interchange of linkage groups" is clearly shown by the appearance of CAM+ and WS— forms and their back recombination to give CAM and WSE. There are, however, significant differences from results with the MEL/WSE system. In the first place, the recombinants are produced much less readily. Instead of finding recombinants forming 20–50 p.c. of the yield, the CAM+ type was isolated only at low dilutions, forming perhaps 1/10,000 of the viable population.

The other difference was that the CAM+ strains all showed moderate pathogenicity for the mouse lung. The formulation of the interaction would be

A′bD-FCE+abd-fce→A′bD-fce+abd-FCE.

If we are right in referring to the genetic determinants of the MEL/WSE system as being distributed in two linkage groups, then we must postulate that WSE in the normal form abdf-ce interacts readily with MEL (ABDF-CE), but in a small proportion it takes the form abd-fce which can then interact with CAM (A′bD-FCE).

In comparing the two reactions, however, it must be remembered that MEL has a definite degree of pathogenicity for the mouse lung, 1AD usually producing 2-3 grade non fatal lesions so that the significance of F in the formulae for

MEL and CAM, respectively, is not quite the same. It is conceivable that minor increase in mouse pathogenicity in the change from MEL to M+, equivalent to the change observed from the wholly inactive CAM to CAM+, could be missed completely. A good deal of weight must, therefore, be placed on the appearance of non mouse pathogenic WS— strains in the present work.

The ease with which the back interaction occurs giving typical nonpathogenic CAM strains indicates strongly that the reaction can be formulated

A′bD-fce+abd-FCE→A′bDFCE+abdfce.

The WSE strains re-formed in this fashion, however, behaved in recombination with CAM precisely as in the first experiment so that one must assume that, however produced, the abdfce complex preferentially splits as abdf-ce.

A further indication of an alternative method by which a gene thread can be broken is given by the appearance amongst the CAM+/WS— progeny of a CAM strain with mouse pathogenicity A′bDfCE. This would presumably arise according to the formula

A′bDf-ce+abdF-CE→A′bDf-CE.

(2) The full recombinants CAM+ and the corresponding WS— were only rarely obtained. Apart from these, the reisolated CAM strains were practically all of the parent type, at most a slight and readily lost pathogenicity for mice was seen in two instances only.

On the other hand, WS strains from double infections with CAM showed a highly significant range of variants in which both mouse pathogenicity f and embryo pathogenicity e might be lost or variably reduced. These changes were not associated with the appearance of corresponding new virulences in CAM strains from the same experiments. This is an example of a finding we have met in other systems, that in most forms of genetic interaction between viruses it is easier to lose virulence than to gain it (Burnet and Lind, 1954b).

(3) WS— forms of abd-FCE type were found only under conditions where secondary recombination might occur. The strain used for the reciprocal recombination experiments was obtained from the lung of an embryo dead without haemorrhages after inoculation with a mixture of undiluted first cycle fluid and anti-WS serum. The lung contained CAM virus in addition to WS—. Other WS— clones of abdf-CE type were found more frequently but only one appeared to be derived directly from the first cycle fluid. In one experiment the same first cycle fluid was titrated, (a) diluted in weak anti-CAM serum, and (b) diluted in normal serum saline, the eggs receiving a dose of 1 : 10 anti-CAM serum 2 hours after the virus dilutions were inoculated. The limit fluids in the first were all WSE, but from titration (b) two had WS— character.

This suggests strongly that heterozygotes, which would be prevented from inducing infection in the presence of anti-CAM serum, could, in its absence, enter cells and give rise there to secondary recombination with the production

of a proportion of WS— derivatives which would be unaffected by the antiserum subsequently added.

(4) As shown in Tables 1 and 7, most of the first cycle fluids with reasonably equivalent inocula showed double neutralization to a significant extent.

There is as yet no accepted basis on which the phenomena of influenza virus genetics can be interpreted, and it seems hardly appropriate to elaborate discussion of the present results before such a basis is available. It is hoped that a reasonably comprehensive review of the work on influenza virus will be completed by one of us within the next year.

Here we should like merely to indicate that we prefer to assume now that the labile virulence genes, which must be postulated to account for the phenomena of redistribution of virulence, are directly associated with other genes in linkage groups or gene threads.

Our present concept is to think of the virus particle as carrying a genetic mechanism which comprises a considerable number (perhaps between 5 and 50) of similar units which, for the sake of convenience, we call gene threads. Each of these carries the complete sequence of genes, e.g., in the case of WSE, abdf-ce. The degree of virulence for the chick embryo in this instance will be determined by the ratio of the number of gene threads carrying the "virulence" allele e to those carrying the corresponding allele for lack of virulence E.

Since we accept Ada and Perry's (1954) evidence that DNA plays no part in influenza virus genetics, we feel that there is no major obligation to consider that any postulated mechanism should be formally equivalent to those for bacterial viruses or for higher organisms in which the role of DNA is predominant. It may well be that the influenza virus system is more analogous to the RNA—controlled system of protein synthesis in the cytoplasm than to any type of chromosomal system.

SUMMARY.

Recombination between the A prime strain CAM and the influenza A strain WSE gave rise to the forms CAM+ and WS— corresponding to an interchange of characters formulated as

A′bD-FCE+abd-fce→A′bD-fce and abd-FCE.

A very small yield only of recombinants was obtained.

Back recombination, according to the scheme

A′bD-fce+abd-FCE→abdfce+A′bDFCE,

occurred readily, giving a high yield of both original forms.

A variety of WS derivatives showing varying degrees of loss of virulence for the mouse lung and/or the chick embryo were obtained in these interactions.

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THE METABOLISM OF THE SEMINAL VESICLE OF THE GUINEA-PIG

3. NUCLEIC ACID CONTENT

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In previous experiments concerning the respiratory activity of the seminal vesicle of the guinea-pig (Humphrey and Robertson, 1953), sub-cellular fractions were isolated and studied separately from each other. As a check on the cytological purity of these fractions, estimation of pentose and deoxypentose was carried out by the methods of Morse and Carter (1949). These analyses were carried out only during the initial stages of the investigation, simply to enable a method of tissue preparation to be chosen so that there was no destruction of nuclei, with subsequent contamination of cytoplasmic fractions. Further, the analyses showed an apparent ratio of pentose to deoxypentose of about 0·25.

Since only purine bound, but not pyrimidine bound sugar reacts in these methods and the standards used were respectively chemically pure ribose and 70 p.c. pure thymus nucleic acid, these results suggest that the ratio of ribonucleic acid (RNA) to deoxyribonucleic acid (DNA) was 0·7, a value more characteristic of lung, spleen or thymus than of a secretory gland (Davidson, 1947). However, no conclusions were drawn about the amounts of RNA or DNA in the seminal vesicle because sugar estimations alone on the trichloracetic acid extract of Schneider (1945) are not an accurate guide to nucleic acid content, especially in a tissue previously not examined for nucleic acids. This work has now been extended by isolating seminal vesicle nucleic acids, analysing them and using several techniques to estimate the variations in concentration of the nucleic acids as affected by age, season, functional activity and castration.

MATERIALS AND METHODS.

Seminal vesicles were removed from animals immediately after death, either from chloroform or a blow on the head. The excised glands were placed in ice-cold 0·9 p.c. NaCl solution, slit longitudinally and the gel washed out. After rinsing in another dish of ice-cold saline, the tissue was blotted dry, rapidly weighed on a torsion balance, homogenized in an all-glass homogenizer in an ice-bath with 4 to 9 volumes of ice-cold isotonic NaCl, KCl or sucrose for

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about 2 minutes, and an aliquot treated with half its volume of cold 30 p.c. trichloracetic acid. The procedure thereafter followed that of Davidson, Frazer and Hutchison (1951), and RNA and DNA fractions were obtained. The nucleic acid content of these fractions was measured using chemical tests for phosphorus (Allen, 1940) and sugar, and by the ultraviolet absorption of the purine and pyrimidine bases. Ribose was measured by the modification of the orcinol test described by Drury (1948), using ribose or xylose as a standard and correcting for contaminating sucrose if necessary. Deoxyribose was measured by the modification of the diphenylamine reaction described by Racker (1952), using as a standard a sample of calf thymus DNA prepared by the method of Gulland, Jordan and Threlfall (1947), containing 9·2 p.c. P and giving only a faint Sakaguchi test. Small samples of purified nucleic acids were isolated from the seminal vesicle according to the methods of Tsuboi and Stowell (1950), and their ultraviolet absorption related to their phosphorus and sugar content.

Ultraviolet absorption was measured with a Beckman spectrophotometer, and approximate corrections were made for the presence of protein breakdown products in the extracts by measuring optical densities at 260 and 280 mμ, with subsequent calculations similar to those of Drury (1948) for pentose and hexose in the one sample.

In some experiments, the seminal vesicle mucous membrane was rapidly separated from the muscle layer by scraping with the back of a scalpel (the process is usually easy and complete in a normal animal) and determinations were made on the two parts separately. The mucous membrane was fractionated in 0·25 M sucrose according to Schneider (1948), and the nucleic acids determined on the fractions. Nuclei were isolated from the whole gland by the citric acid method of Marshak (1950) and the acetic acid method of Mauritzen, Roy and Stedman (1952).

Fructose was determined by the method of Roe, Epstein and Goldstein (1949) as modified by Hers, Beaufays and de Duve (1953), citric acid by the method of Taylor (1953), and total nitrogen by a micromodification of the Kjeldahl digestion method of McKenzie and Wallace (1954).

RESULTS.

Properties of nucleic acids.

The extinction coefficients per mole P per litre at 260 mμ, pH 7·2, 1 cm. light path, after treatment similar to the whole tissue extracts, were RNA 10,230 and DNA 9,240. The ratio of the orcinol-reacting pentose to P for RNA was 2·6, and the deoxyribose to P ratio for DNA was identical with the standard calf thymus preparations. All results, no matter how obtained, are expressed in terms of the equivalent P, by calculations based on the above figures.

TABLE 1.

Comparison of Methods of Estimating Nucleic Acids

Method	RNA-P (μg per gland)	DNA-P (μg per gland)
Phosphorus	842	304
Sugar	798	295
Ultraviolet	844	287
Mean Value	828	295

The agreement among the three methods used was investigated and some results are shown in Table 1, where all figures are expressed in terms of the equivalent P. In assessing the nucleic acid content of the glands of any particular animal, the mean of the three results is taken. In few cases does any single figure differ by more than 5 p.c. from this mean. Where smaller aliquots were available, only two methods of analysis were used.

Effect of season on adult animals.

Initial results on adult animals (July, 1953) showed that the ratio of RNA to DNA was about 3 or 4, instead of the 0·7 previously found. It was noticed that the glands were much larger, and contained more gel than those on which the previous results were obtained (February, 1951). Accordingly the seasonal variation was examined during the next year. The total gland weight (excluding gel) in the adult animal, of body weight 800 gm. or greater, was found to average 1·60 gm., with standard deviation 0·25 gm., except in the months July to September, when the mean was 2·01 gm.±0·53; the only occasions on which the combined weight of the two glands exceeded 2 gm. were during these three months (7 out of 14 animals). The gel content was from 2 to 5 gm. per animal in the latter group, and rarely exceeded 2 gm. during the rest of the year, with frequently less than 1 gm. The nucleic acid concentration was fairly constant all the year round; the RNA-P ranged from 40 to 60 mg. p.c. and the DNA-P from about 14 to 22 mg. p.c. The seasonal means are shown in Table 2.

TABLE 2.

Nucleic Acid Content of Seminal Vesicle of Normal Adults at Different Seasons.

Months	Number of Animals	RNA-P (mg p.c.)	DNA-P (mg p.c.)	RNA-P/ DNA-P
Jan.-Mar.	5	45·2±10·5	16·2±2·6	2·8
Apr.-Jun.	3	51·8±8·5	20·2±4·2	2·7
Jul.-Sep.	14	49·8±8·5	17·0±3·6	2·9
Oct.-Dec.	4	50·8±6·0	18·3±1·9	2·8
Mean	26	50·0±8·0	17·5±3·4	2·8

Effect of age.

As is shown in Fig. 1a, the weight of the seminal vesicles is constant at about 0·05 gm. until the body weight reaches 200 to 250 gm., and then rapidly increases, reaching about 0·75 to 1·0 gm. at 500 gm. body weight, when sperm are first found in the epididymis. After this the rate of increase tends to slow, but the total weight may reach 2·75 gm. in certain months. The weight of the separated mucous membrane increases from about 0·08 gm. at 300 gm. body weight to 0·5 to 1·0 gm. in large adults.

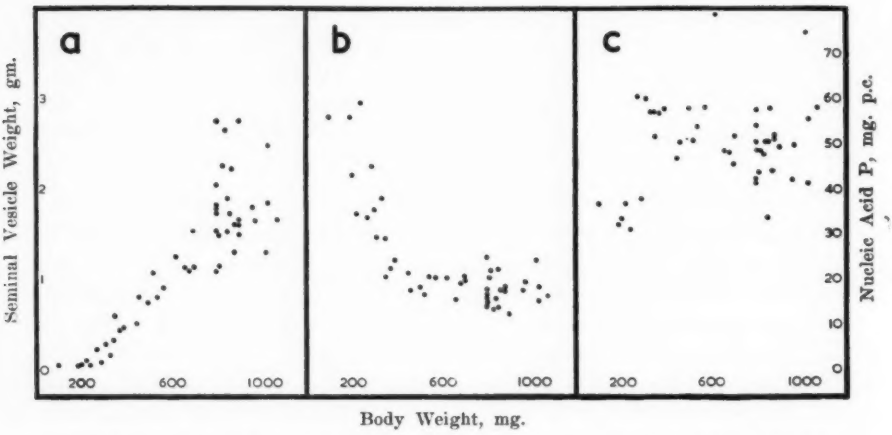

Fig. 1. Variation of seminal vesicle weight and nucleic acid content with body weight.
a. Seminal vesicle weight, gm.
b. DNA phosphorus, mg. p.c.
c. RNA phosphorus, mg. p.c.

In the very young animal (less than 200 gm. body weight) the DNA-P content is 55 to 60 mg. p.c. During the rapid growth period the DNA-P content falls to 15 to 20 mg. p.c., at which value it remains during the subsequent slower growth (Fig. 1b).

In the very young animal the RNA-P content is about 30 mg. p.c. With the commencement of the period of rapid growth, at about 300 gm. body weight, it increases suddenly to the adult value of 50 to 60 mg. p.c., and remains there or even falls slightly with increasing size (Fig. 1c). This sudden increase coincides with the appearance of small quantities of gel, which contains the adult concentration of both fructose (150–180 mg. p.c.) and citric acid (300 to 400 mg. p.c.).

The season of the year appears to have no effect on the rate of development of the glands in the young animals.

Distribution of nucleic acids in the gland.

The normal gland is a tubular structure, with an outer layer of smooth muscle, covered externally by peritoneum and lined by the mucous membrane. The latter has a villous structure, somewhat resembling the small intestine; its columnar epithelial cells have fairly large nuclei in the basal half.

The DNA-P of the muscle layer is low; from about 20 mg. p.c. at 300 gm. body weight it falls rapidly to about 8 mg. p.c. at 500 gm. body weight, and then more gradually to about 6 mg. p.c. in the adult. The RNA-P falls from about 30 mg. p.c. at 300 gm. body weight to about 18 mg. p.c. at 500 gm. body weight, and remains fairly constant with continued growth.

Both types of nucleic acid are much more abundant in the mucous membrane. The DNA-P content ranges from 40 to 60 mg. p.c., and the RNA-P from 120 to 160 mg. p.c., in all animals in which the separation can be performed. It appears that a good deal of variation, seen in Fig. 1, between animals of similar weights, and even of widely differing weights, may be explained by different proportions of the two tissues, each of a relatively constant composition.

In Table 3 are shown the results of a typical cell fractionation experiment on the mucous membrane. The tissue was homogenized in 0·25 M sucrose for 3 minutes, centrifuged at 2,000 *g* for 2 minutes, and the sediments re-homogenized in fresh sucrose for 2 minutes. The process was repeated, and the final homogenate added to the two supernatants to give the "whole homogenate." This was fractionated according to Schneider (1948); the microsome fraction was sedimented at 25,000 *g* for 90 minutes, and was not washed. Results of phosphatase distribution and direct mitochondrial counts (to be published later) suggest that about 65 p.c. of the tissue mitochondria were isolated in the mitochondrial fraction, the rest being equally divided between nuclear and microsomal fractions, and that about 75 p.c. of the microsomes were isolated in the microsomal fraction, the rest being equally in nuclear and mitochondrial fractions, with only 2–3 p.c. in the "soluble" fraction (judged by subsequent sedimentation at 100,000 *g* for 30 minutes). Experiments on nuclei isolated in citric or acetic acid showed RNA to DNA ratios of about 0·25 to 0·40.

TABLE 3.

Composition of Subcellular Fractions of Seminal Vesicle.

Fraction	DNA-P (p.c. of whole homogenate)	RNA-P (p.c. of whole homogenate)	Total N (p.c. of whole homogenate)
Nuclei	90	25	25
Mitochondria	6	7	6
Microsomes	0	30	12
Soluble	0	30	45
Total Recovery	96	92	88

Effects of functional activity.

All of the above results were obtained on segregated male animals. Animals which had been kept up to a month in a breeding pen with several females frequently showed almost empty glands, with marked thinning of the mucous membrane, which was quite friable and difficult to scrape off completely. The mean gland weight in 20 animals was only 1·27 gm.±0·25 (normal 1·8 gm.±0·4), and whereas the mean RNA-P concentration was normal (51·0 mg. p.c.±9·4), the mean DNA-P was higher than normal (23·5 mg. p.c.±6·1 instead of 17·5

mg. p.c.±3·4). There was also marked variation in the DNA-P content within the group; glands macroscopically normal showed normal DNA-P content, whilst high DNA-P content was found in the thin, friable mucosa. The RNA to DNA ratio fell as low as 1·5 in two cases, and the group mean was only 2·1, the normal being 2·8. Separated mucous membrane showed normal RNA-P content of about 130 mg. p.c., but the DNA-P content was as high as 110 mg. p.c., due to loss of cytoplasm with preservation of nuclei. This accounts for the high DNA content of the whole gland.

Effects of castration.

In animals castrated 6 to 8 months previously, there was a decrease in total gland weight to about 0·4 gm., this including some fatty infiltration, with RNA-P and DNA-P both about 30 mg. p.c. This means a decrease in total DNA-P from about 300 μg. per animal to about 120 μg. per animal, i.e., a loss of about 60 p.c. of the cells. It is not possible to scrape sufficient mucous membrane off the muscle for accurate analysis in animals castrated for longer than 3 to 4 weeks. During that time the muscle weight decreases by about 50 p.c., and the mucous membrane decreases from about 0·5 gm. per animal to about 0·05 gm. per animal. As is seen in Fig. 2, the DNA-P increases within a week after castration to more than 100 mg. p.c. and then tends to decrease, while the RNA-P decreases gradually to about half the normal value. No figures are available for the muscle nucleic acids, as it is not possible to remove the extremely friable mucous membrane completely in these animals.

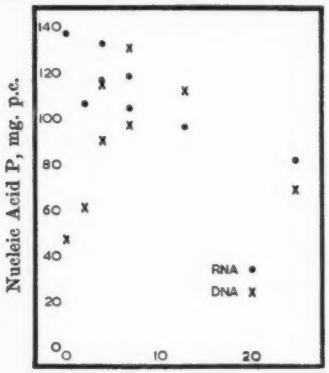

Fig. 2. Effect of castration on nucleic acid content of seminal vesicle mucous membrane.

DISCUSSION.

The values found for the extinction coefficients of the seminal vesicle nucleic acids show good agreement with those given by Tsuboi and Stowell (1950) for mouse liver nucleic acids, being only slightly lower; for RNA, 10,230 compared with 10,760 and for DNA 9,240 compared with 9,520. The sugar to phosphorus ratios are the same as found with other mammalian nucleic acids, and hence the total purine to total pyrimidine base ratios are also similar to other mammalian nucleic acids. The agreement among the three analytical methods used for the determination of nucleic acids in the tissues was good, the biggest discrepancy

being about 5 p.c. from the mean (Table 1). The RNA to DNA ratio for the animals used to obtain these results was about 3, much greater than the value previously found. The previous series of analytical results was obtained during the beginning of the year on old males which had small seminal vesicles containing very little secretion, compared with somewhat younger adults used during the succeeding 15 months of the investigation. Therefore, in this present investigation it was thought advisable to determine the variation in nucleic acid content among animals at different times of the year, at different ages and also of different sexual activity. The results obtained have not shown that adults possess glands whose ratio of RNA to DNA is as low as 0·7, but they have shown that the ratio is markedly affected by age and sexual activity. For example, in very young animals the ratio is as low as 0·5, and if they are allowed to grow up while they are segregated from females the ratio reaches 3 or 4. After a few weeks in a pen with females, the ratio falls to about 2. When the previous investigation was commenced, the initial experiments were performed on the largest animals; these were probably senile and this may account for the emptiness and smallness of their glands and the low RNA to DNA ratio. However, there is no certainty of this because histories were not kept for the animals at that time, and no very old males were available for the present series. The concentration of RNA and DNA found in the present series of normal adults is very similar to the values given for rat liver, sheep liver, heart muscle, rat kidney, sheep testis (Davidson, 1947), but somewhat lower than pancreas, a more actively secreting gland.

During the growth of the glands, the DNA of the muscle slowly doubles in absolute amount, but it is not known whether this is due to cell multiplication or to polyploidy. The muscle itself increases from about 0·2 to 1·6 gm., each fibre increasing markedly in size; the RNA concentration is slightly higher during the most rapid growth and then it settles down to a fairly constant figure. No results are available for the very small glands with less than 0·2 gm. muscle as it is not possible to separate their mucous membrane. However, the secretory tissue has relatively constant RNA to DNA concentrations from the earliest time at which it can be separated, and this fact, in conjunction with the constant composition of the secretion (in terms of fructose and citric acid) suggests that the functional cell is a fairly constant entity, growth in the gland being accompanied by a considerable increase in cell number.

In the experiments on cell fractionation, it was difficult to obtain clean nuclei; examination with the phase contrast microscope at a magnification of 500 revealed that at least 10 p.c. of the nuclei had cytoplasmic tags about the size of mitochondria, and therefore the RNA to DNA ratio of 0·25 to 0·40 must be regarded as a maximum. The quantities of tissue available were so small that the more rigorous procedures of purification described by other authors, involving discarding the greater portion of the nuclei after each washing,

were not practicable. The distribution of the nucleic acids among the subcellular fractions (Table 3) shows quite clearly that the nuclei have not been significantly broken during homogenization. The figures for RNA and total nitrogen are similar to those given by Schneider (1948) for fractions from rat liver, except that the mitochondrial fraction from the seminal vesicle is relatively small. This difference is being examined further and will be discussed in a later publication.

SUMMARY.

The extinction coefficients per mole P per litre at pH 7·2, 260 mμ and 1 cm. light path are 10,230 for seminal vesicle RNA and 9,240 for DNA.

In young animals (less than 200 gm. body weight), RNA-P is 30 mg. p.c. and DNA-P is 60 mg. p.c. In adults kept from females, the gland averages 1·6 gm. with 2 gm. gel except in July to September, when the gland average is 2 gm. and the gel constant as high as 5 gm. The RNA-P is 50 mg. p.c. and the DNA-P 18 mg. p.c. throughout the year. If the adults are kept in a cage with females, the glands weigh only 1·3 gm., often contain very little gel, and although the RNA-P is normal the DNA-P rises to 24 mg. p.c., due to loss of cytoplasm with preservation of nuclei. In animals castrated 6 to 8 months previously the glands weigh only 0·4 gm. and RNA-P and DNA-P are both 30 mg. p.c.

The concentration of each nucleic acid in the mucous membrane is about 8 times that in the muscle. The DNA is in the gland nuclei exclusively, and the RNA is equally distributed among the nuclei, microsomes and soluble constituents, the mitochondria containing no more than 10 p.c. of the total RNA.

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SOME OBSERVATIONS ON THE INCREASE IN THORACIC DUCT LYMPH FLOW DURING INTESTINAL ABSORPTION OF FAT IN UNANAESTHETIZED RATS

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(Accepted for publication 17th November, 1954.)

In earlier experiments (Simmonds, 1954) in unanaesthetized rats it appeared that fluid and fat alone were responsible for the increase in thoracic duct lymph flow after a meal. In these experiments mixed foodstuffs and pure protein, carbohydrate or fat were washed into the stomach with fairly large volumes of fluid. The conclusion that fat but not carbohydrate or protein increased lymph flow has now been verified by feeding larger amounts of food with small amounts of fluid. The object of this paper is to report the results of such experiments, together with serial observations on the changes in lymph flow and protein and fat concentrations in the lymph during the absorption of fat and fatty acid. The latter confirm and extend in some particulars the results of Borgström and Laurell (1953).

METHODS.

Male rats of an albino Wistar strain were used in all experiments. The weights varied from 200 to 330 gm., and were usually about 250 gm.

Operative and postoperative treatment. The abdominal thoracic duct was cannulated, under ether anaesthesia, by the method of Bollman, Cain and Grindlay (1948), a gastrostomy tube inserted, the wound sutured and the animal placed in a restraining cage of the type devised by Bollman (1948). The postoperative treatment, collection of lymph and care of fistula have been previously described (Simmonds, 1955).

Experimental procedure. Experiments began at 9 a.m. on the morning following operation, that is after a post-operative period of 16–20 hours. No food was given other than that used in experiments. The fluid intake was controlled, as previously described, Ringer-Locke solution being administered continuously at 0·5 ml. per hour through the gastrostomy tube. An extra 5 ml. was given at the end of a 10-hour fat absorption period, to replace some of the extra lymph produced. Each experimental session consisted of three periods. In the first, from 9 a.m. to 11 a.m. or later, lymph was collected to establish a baseline for lymph flow and fat and protein concentrations in lymph. When a steady rate of flow had been maintained for 1 to 2 hours the second period commenced. This lasted 10 hours.

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[1]This work was performed with the aid of a grant from the National Health and Medical Research Council, Canberra.

In some experiments, olive oil, oleic acid or tributyrin, 0·5 ml., was injected into the gastrostomy tube by syringe at the beginning of the period, and flushed through with about 1 ml. of Ringer-Locke solution. In other experiments the period began when access was given to a weighed amount of foodstuff, and, on some occasions, further meals were given at intervals throughout the period. Lymph flow was measured at half-hourly intervals, protein and fat concentrations being determined in the lymph collected in successive hours for the first 6 hours and then 2-hourly for the remaining 4 hours. The third period then followed, lasting from 9 or 10 p.m. until 9 o'clock on the next morning. In most rats a second experimental session then commenced, conducted in the same way as on the first day.

Substances administered. Fat. A dose of 0·5 ml. olive oil (B.P.), oleic acid, redistilled (B.P.), or tributyrin (B.D.H.) was washed into the stomach through the gastrostomy tube with not more than 1 ml. of Ringer-Locke solution. *Foodstuff.* The initial and subsequent meals consisted of 1 gm. in all cases, made up to a paste with about 1 ml. of water. The fat-free food used was skim milk powder (fat 1 p.c., protein 36 p.c., carbohydrate 52 p.c.). The fatty food tested was full cream milk powder (fat 26 p.c., protein 28 p.c., carbohydrate 38 p.c.) 1 gm., or skim milk powder plus olive oil, 1 gm. and 0·25 ml. respectively.

Analytical. Total nitrogen and N.P.N. concentrations in lymph were determined by micro-Kjeldahl digestion and direct Nesslerization, using a Spekker photoelectric absorptiometer. N.P.N. was determined after precipitation of protein with trichloracetic acid. Total esterified fatty acid concentration in lymph was determined by the method of Stern and Shapiro (1953).

Opacity of lymph. Optical densities of lymph diluted 1 in 26 with 0·9 p.c. NaCl solution were measured at 500 and 650 mμ on a Beckman spectrophotometer, against 0·9 p.c. NaCl solution.

RESULTS.

The effect of fat in mixed foodstuffs on lymph flow. In 5 experiments in 4 rats a foodstuff containing virtually no fat—powdered skim milk—had no pronounced or consistent effect on lymph flow. Individual rats ate from 1 to 4

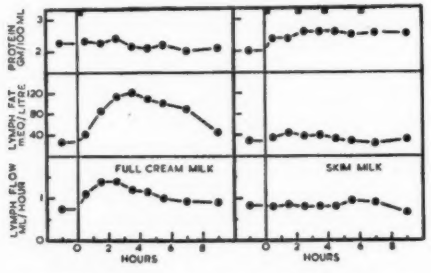

Fig. 1. Change in lymph flow and in fat and protein concentration in the lymph in the same rat after full cream milk powder and skim milk powder, given on successive days. Time of meals, consisting of 1 gm. powder and 1 ml. water, and approximate time taken for their consumption are indicated by bars at top of diagram.

meals each of 1 gm. without changes in flow or composition of lymph attributable to individual meals or to a cumulative effect of feeding. The results of the experiment shown on the right hand side of Fig. 1 are typical of the 5 in the series. This rat had a most vigorous appetite, consuming each meal in less than half an hour except the last, which was half eaten, and any considerable effect of protein or carbohydrate digestion should have been apparent.

On the left hand side of Fig. 1 can be seen the very different results when only 1 gm. of powdered full cream milk was eaten by the same rat. The increase in lymph flow, associated with increasing concentrations of fat in the lymph and lasting for a number of hours, together with the absence of changes in protein concentration in the lymph occurred in all 3 experiments in this series. A larger number of tests was not performed since there was little doubt, from previous experiments and from observations in the literature, that fat absorption was accompanied by increased lymph flow in unanaesthetized rats. The experiments with large amounts of skim milk, on the other hand, gave further support to the conclusion (Simmonds, 1954) that digestion and absorption of carbohydrate and protein have little or no effect on thoracic duct lymph flow in rats.

The relationship between lymph flow during fat absorption and the concentrations of fat and protein in the lymph. Three rats were given 0·5 ml. olive oil and another 3 given 0·5 ml. oleic acid, in all cases on the day following operation. The average changes in lymph flow and composition of the lymph are shown in Fig. 2.

With both olive oil and oleic acid, an increasing lymph flow was associated with an increasing fat concentration in the lymph, the regression of lymph flow on fat concentration being statistically significant ($p<0·02$ and $p<0·001$ for oil and acid respectively) during this period. However, the lymph flow reached its highest rate some 3–4 hours before maximum fat concentrations in the lymph

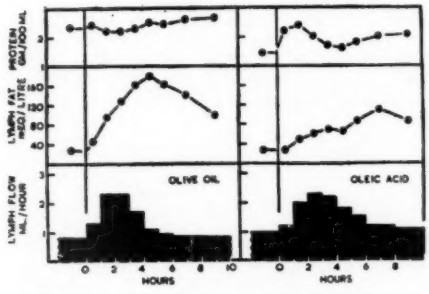

Fig. 2. Average effects of 0·5 ml. olive oil and 0·5 ml. oleic acid, 3 rats in each group, on lymph flow and on fat and protein concentration in the lymph.

were attained, and returned to preabsorptive rates while lymph fat concentrations were still high. In addition to the general similarity between the effects of olive oil and oleic acid there were differences in detail. For example, the lymph flow increased and decreased more slowly after oleic acid administration and the lymph fat concentration increased much more slowly. It also appeared that, after oleic acid, a given increase of fat concentration was associated with a larger increase of lymph flow than after olive oil ($p<0{\cdot}05$ for difference in slope of regression lines). Evaluation of this finding is difficult since the rats which received oleic acid had a somewhat higher preabsorptive lymph flow than the group given olive oil.

It is undoubtedly significant that the protein concentration in lymph did not fall below preabsorptive values during the increased lymph flow after olive oil and oleic acid. The averaged curves in Fig. 2 are representative of the results in individual rats. Protein concentrations after oleic acid were more variable than after olive oil, but in all 3 rats in the former group there was a considerable increase in protein concentration in the first hour after administration of oleic acid, when little or no fat absorption had occurred. It must be mentioned, however, that in 2 of these 3 rats preabsorptive lymph flows were higher and protein concentrations lower than in any of the 3 which received olive oil. It can be seen, in Fig. 3, that the pattern of protein *output* after oleic acid was almost identical with that after olive oil.

Comparison of Fig. 3 with Fig. 2 shows that there was a much closer correspondence between increasing fat output in the lymph and increasing flow

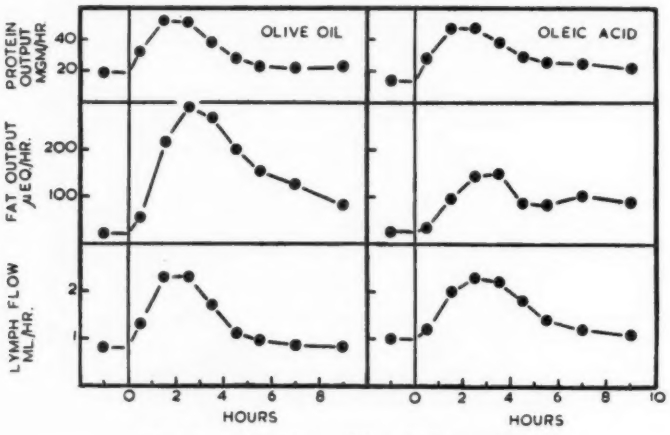

Fig. 3. Comparison of lymph flow and fat and protein outputs in lymph after 0·5 ml. olive oil and 0·5 ml. oleic acid; same experiments as in Fig. 2.

than between increasing fat concentration and flow. The fat output in the lymph should give some indication of the rate at which fat was being extruded from the intestinal epithelium into mucosal tissue fluid. The correlation of lymph flow with this phase of fat absorption was by no means complete. After olive oil, maximum lymph flows were attained significantly earlier than maximum fat outputs. Thus, between the 2nd and 3rd hour there was a mean increase in flow of 1·66±7·10 p.c. while the mean increase in fat output was 33·3±3·83 p.c., the difference being statistically significant ($p<0·02$). In the case of oleic acid there was only a small decrease in fat output from the 3rd to 10th hours, while lymph flow declined to its preabsorptive value.

The data on changes in lymph flow, serial fat concentrations and fat outputs in the lymph after dyed olive oil and dyed oleic acid (Simmonds, 1955) were in accord with the present findings.

The relationship between opacity of lymph and fat concentration. Borgström and Laurell (1953) found that the ratio of optical density, at 500 mμ, of diluted lymph to the fat concentration in the lymph increased for several hours during fat absorption and then remained fairly steady. They considered that this indicated an increase in the mean diameter of the fat particles during the early stages and thereafter a relatively constant size which, they thought, might have been related to changes observed in the ratio of phospholipid to total fat. Fig. 4 shows the mean ratios of optical density, at 500 and 650 mμ, to fat concentration at various times after feeding olive oil and oleic acid. These observations confirm those of Borgström and Laurell. Their suggestion concerning changes in mean size of the chylomicrons received support from the results of plotting optical density of diluted lymph against fat concentration in individual lymph samples. The optical density of the lymph increased less for a given increase

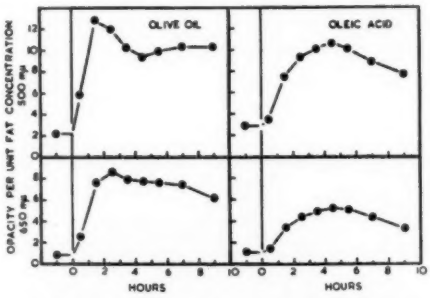

Fig. 4. Changes in average ratio, in arbitrary units, of optical density of lymph, diluted 1:26, to fat concentration in undiluted lymph, after 0·5 ml. olive oil and 0·5 ml. oleic acid. Same experiments as in Figs. 2 and 3. Arbitrary units=O.D×1,000: Fat in meq./l.

in fat concentration after the first 2–3 hours, the slopes of the regression lines being significantly different. This would be consistent with a smaller increase in the number of particles for a given increase in fat concentration, that is with larger particles. Comparison of the results after oleic acid with those after olive oil suggested that serial estimations, by more direct methods, of the size and composition of the chylomicrons after feeding these two substances might be of considerable interest.

Effect of ingestion of tributyrin. Work with radioactive fats has shown that the proportion of absorbed fat carried away in the lymph diminishes with decreasing length of the fatty acid chain (Bloom, Chaikoff and Reinhardt, 1951). After feeding tributyrin none appears in the lymph (Hughes and Wimmer, 1935) or in body fat depots (Eckstein, 1929). This short-chain triglyceride is well tolerated in small doses, probably completely hydrolysed, and readily absorbed from the intestines in rats. In 7 experiments in 4 rats, ingestion of 0·5 ml. tributyrin was followed by an increased rate of lymph flow. However, in contrast with the effects of olive oil, the protein and fat concentrations in the lymph decreased and there was no significant increase in fat output. Fig. 5 shows the average results of the 7 experiments. Three of the rats received tributyrin on two successive days and the other was fed only on the first post-

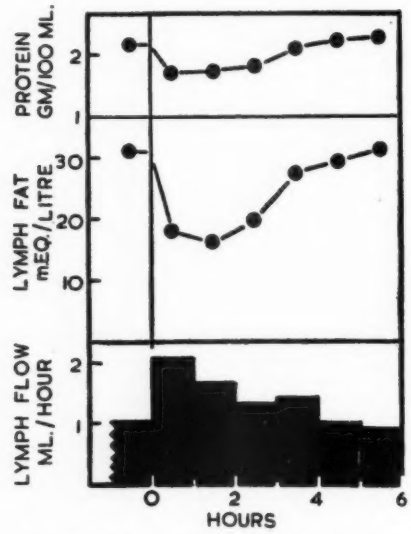

Fig. 5. Effect on lymph flow, fat and protein concentrations in lymph of 0·5 ml. tributyrin; average of 7 experiments in 4 rats.

operative day. The changes were much the same in all 4 rats and the second test gave results very similar to the first.

Tributyrin, therefore, produced a considerable increase in lymph flow but the pattern of changes in composition was quite different from that after fat which was destined to be transported by the lymph. There was no evidence clinically, such as restlessness or loose motions, nor macroscopically at post-mortem that tributyrin caused gastrointestinal irritation in the dosage used. The difference in pattern of lymph composition suggests that the mechanism of increase of lymph flow is different from that after feeding olive oil. Direct observations of the changes in mucosal motility and blood flow may be of use in testing this suggestion.

DISCUSSION.

In the course of these experiments and of others previously reported it became increasingly apparent that fat absorption was accompanied, in unanaesthetized rats, by a specific and somewhat unusual increase in lymph formation and flow. This was specific in the sense that a food consisting almost entirely of carbohydrate and protein, namely skim milk, produced little or no change in lymph flow while pure carbohydrate and protein in solution produced only changes consistent with the volume and tonicity of the fluid (Simmonds, 1954). It should be mentioned, however, that Cain, Grindlay, Bollman, Flock and Mann (1947) found, in unanaesthetized dogs, that the post-prandial increase in lymph flow did not depend on the type of meal.

The increase in lymph production during fat absorption in rats was of an unusual type in so far as the lymph protein concentration was virtually unaltered; except when fairly large amounts of free fatty acid were fed, which was followed by a rise in protein concentration. The absence of a fall in protein concentration despite very large increases in flow suggests that the increased flow was not due to fluid absorbed from the lumen of the intestine (Simmonds, 1954). Similarly, a lowered protein concentration with increased lymph flow has been reported in circumstances in which filtration from blood capillaries is believed to have been increased without significant change in their permeability to protein (McCarrell, 1940; Courtice, 1946). On the other hand, a considerable rise in lymph protein concentration—to levels approaching those in plasma—has been found in many instances when the capillary endothelium has been definitely injured (Field, Drinker and White, 1932; Cope and Moore, 1944; Cameron, Courtice and Short, 1947). Capillary damage may be excluded in the present experiments. It is by no means certain, despite a considerable amount of work recorded in the literature, that intermediate grades exist between the gross leakiness to protein of the damaged capillary and the somewhat variable but still very restricted escape of protein from normal capillaries. It might be

suggested that the volume of capillary filtrate could be increased without alteration in its protein concentration or in the proportion of the filtrate reabsorbed, without protein, into the blood vessels. The turnover of tissue fluid and lymph might then be increased without change in their composition. This might occur with hyperemia giving an increased capillary area if the balance of pressures were not greatly affected. It might also occur, under certain circumstances, if tissue fluid were forced more rapidly into the capillary lymphatics. An attempt is being made, therefore, to see if fat absorption has a specific effect on the vascular pattern and blood flow in the mucosa or on its motility. It seems unlikely that potential changes in the composition of the tissue fluid and lymph were masked by the fluid and protein already in these compartments (Wasserman and Mayerson, 1952; Korner, Morris and Courtice, 1954), since the changes in lymph flow were quite abrupt and the volume of lymph collected in 10 hours after 0·5 ml. of fat was 2 to 3 times the plasma volume or more. The small, early rise in protein concentration noted by Borgström and Laurell (1953) after 1 ml. corn oil, and ascribed by them to shrinking of extra-cellular fluid associated with secretion into the intestinal lumen, was not seen in the present experiments. This was probably due to the small amount of fluid used to wash through the dose of olive oil, since control experiments showed a small decrease in lymph protein concentration and a small increase in lymph flow in the first hour after giving 1 ml. of Ringer-Locke solution. The significance of the considerable increase in protein concentration after oleic acid cannot be assessed at present.

It is difficult to correlate the changes in lymph flow with definite events in the absorption of fat. The close relationship between the onset of increased flow and a milky appearance of the lymph after both olive oil and oleic acid suggested that digestion of fat played little part. Of the other three main stages—passage into the epithelial cell, metabolic changes within the cell and expulsion from the cell into interstitial fluid—it seems unlikely from Borgström and Laurell's findings, which were confirmed in the present experiments, that the rate of expulsion of fat from epithelial cells into tissue fluid and the rate of lymph flow are closely linked. As previously mentioned, increasing lymph flow was significantly related statistically to increasing concentration of fat in the lymph and, presumably, in tissue fluid; but maximum lymph flows were reached some hours before maximum lymph fat concentrations. The lag seemed too great to be explained on mechanical grounds. Again, as Fig. 3 shows, changes in lymph flow followed changes in lymph fat output fairly closely after olive oil. The delay between events in tissue fluid and changes in the lymph might have been partly responsible for the observed lag in fat output. However, the discrepancies, especially after oleic acid, make it more likely that changes in lymph flow are linked with some stage in the treatment of fat before it is expelled from the epithelial cell. Presumably the changes in lymph flow are mediated by some humoral effect on mucosal blood flow or motility, or on both.

SUMMARY.

Serial changes in the rate of flow and in the composition of thoracic duct lymph were measured after feeding 1 to 4 gm. powdered skim milk, 1 gm. powdered full cream milk, and olive oil, oleic acid and tributyrin, each in doses of 0·5 ml., to unanaesthetized rats.

Digestion and absorption of protein and carbohydrate in skim milk, even when large amounts were eaten, produced no significant changes in composition or flow of lymph. Fat, on the other hand, whether pure or in mixed foodstuff, produced a considerable increase in lymph flow.

The lymph flow increased concurrently with increasing fat concentrations and fat outputs after full cream milk, olive oil and oleic acid, suggesting that absorption rather than digestion of fat determined the flow. Discrepancies between the curves for fat concentration and fat output, on the one hand, and lymph flow on the other, suggested that the lymphagogue effect was related to some event preceding expulsion of fat into the intestinal tissue fluid. The lymph protein concentration remained unchanged after full cream milk and olive oil. The implications of this finding in regard to the mechanism of the increased lymph flow are discussed.

Tributyrin did not appear in the lymph but produced an increase in flow accompanied by a fall in protein concentration.

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A MUCOPROTEIN FROM BOVINE SUBMAXILLARY GLANDS WITH RESTRICTED INHIBITORY ACTION AGAINST INFLUENZA VIRUS HAEMAGGLUTINATION

by C. C. CURTAIN AND JOHN PYE[1]

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Inhibitors of influenza virus haemagglutination have been found to be widely present in the tissue and secretions of various mammalian species (Anderson, 1950). There are no known exceptions to the rule that all such inhibitors active to high titre are of mucoprotein composition and several have been obtained in purified form—from human urine (Tamm and Horsfall, 1952), sputum (Marmion, Curtain and Pye, 1953), and from sheep submaxillary gland extracts (McCrea, 1953). Most of the inhibitors are highly active against all influenza viruses in the indicator phase and to a minor degree against active virus. It is consistently found that amongst the active viruses available, PR8 is most susceptible to inhibition by mucoprotein inhibitors. Sheep salivary mucin is without action on the strain MEL of influenza A irrespective of whether the latter is in the active or indicator phase (Stone, 1949b), but acts as a typical inhibitor of all other influenza virus strains in the indicator phase.

Though some indications had been obtained previously that bovine mucins might be inactive as inhibitors, an extract of bovine submaxillary gland was found to show activity only against indicator PR8. It was thought possible that a comparison of purified material from the bovine glands with the corresponding inhibitor from sheep, which has been extensively studied by McCrea, might throw some light on the relation between chemical nature and biological specificity.

This paper describes the isolation from bovine submaxillary glands of a mucoprotein (B.S.M.) which is an active inhibitor of haemagglutination by indicator PR8, but has a trivial or no action on other viruses. Evidence will be given that most of the "receptors" are inaccessible to most of the influenza viruses but are accessible as substrates for the enzymic action of the receptor destroying enzyme (RDE) from *V. cholerae*.

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MATERIALS AND METHODS.

Submaxillary glands. Submaxillary glands were collected from sheep and cattle freshly slaughtered at the Melbourne City Abattoirs. The glands were chilled to 0° C. not more than 15 minutes after collection and were processed within 24 hours.

Normal saline. 0·147 M A.R. sodium chloride in distilled water.

Calcium acetate buffer, pH 6·0. 0·04 M acetic acid, 0·96 M sodium acetate, 0·05 M calcium chloride—diluted to the desired ionic strength.

Calcium borate buffered saline. 0·143 M sodium chloride, 0·01 M calcium chloride, 0·02 M boric acid, 0·0025 M sodium borate.

Influenza viruses. The following strains were used in the form of infected allantoic fluids harvested from chick embryos inoculated at the age of 11 days and incubated for 40 hours at 35° C.

Influenza A. MEL (Melbourne), WSE (egg adapted strain of WS), PR8, BEL and Ocean Island.

Influenza B. LEE, BON and MIL B.

Influenza C. Taylor's 1233 strain.

Also Newcastle disease of fowls and swine influenza (NDV and Swine).

Indicator viruses were prepared by the methods of Stone (1949a).

Purified viruses were prepared from the infected allantoic fluids by adsorption to and elution from red cells followed by high speed centrifugation. Details have been given by Gottschalk and Perry (1951).

Purified receptor destroying enzyme of Vibrio cholerae (*RDE*) was prepared by the method of Ada and French (1950).

Electrophoresis was carried out at 2° C. in a Tiselius apparatus with a Philpot-Svensson optical system (Holden, Ada and Pye, 1952) using either the 11 ml. or 2 ml. fused "Pyrex" cell.

A potassium phosphate buffer was used, pH=6·9, ionic strength, μ=0·2; 0·091 M KH_2PO_4, 0·055 M KOH.

EXPERIMENTAL.

McCrea (1953) has reported the isolation of an electrophoretically homogeneous inhibitor from sheep submaxillary glands. The method used, however, seems to be unsuitable for the purification of material for chemical and physical studies since it involves heating the crude mucin to 80° C. and repeated chloroform-butyl alcohol extractions. It has been noted (Curtain, 1953; Marmion, Curtain and Pye, 1953) that inhibitory mucins bind the alkaline earth cations, particularly barium, and this property considerably simplifies fractionation procedures with organic solvents.

All procedures were carried out as close as possible to 0° C.

Extraction of mucins from bovine submaxillary glands. The glands were dissected free of fat and connective tissue and homogenized in a Waring blendor for three minutes with twice their weight of distilled water. To eliminate heating it was found advantageous to reduce the speed of the blendor with a variable voltage transformer. The extract was centrifuged at 5,000 *g* for 20 minutes, the supernatant adjusted to pH 4 with 5 N acetic acid and the resulting

precipitate removed by centrifugation (5,000 *g*, 20 mins.). The supernatant was neutralized with 5 N sodium hydroxide and dialysed against two changes of excess distilled water for 48 hours. This dialysed extract was then fractionated with methanol.

Methanol fractionation. The dialysed extract was made 0·1 M with respect to barium by adding solid barium acetate. To this solution, methanol, pre-cooled to —40° C., was added with stirring to give an alcohol concentration of 64 p.c. (v/v). The precipitate which formed was recovered by centrifugation (2,500 *g*, 15 mins.), dissolved in an 0·1 M solution of ethylene-diamine-tetra-acetic acid (disodium salt) (EDTA) and was dialysed for 60 hours against three changes of excess distilled water. The solution was then refractionated under identical conditions except that the methanol concentration used was 56 p.c. (v/v).

Electrophoretic behaviour of B.S.M. inhibitor.

The final product was submitted to electrophoresis in a phosphate buffer (pH 6·9, μ=0·2). In most cases a single peak was obtained with a mobility of $-5{\cdot}4\times10^{-5}$ cm^2. sec^{-1}. volt^{-1} (Fig. 1), but some preparations gave evidence of considerable heterogeneity. This was found most likely to occur when the temperature was allowed to rise during the purification, particularly in the blending stage.

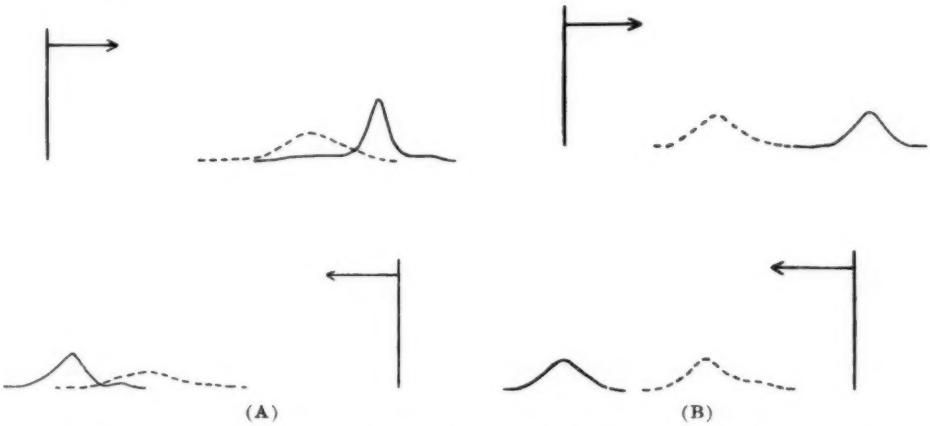

Fig. 1. Electrophoresis in phosphate buffer (pH 6·9, μ=0·2) of an 0·4 p.c. (w/v) solution of the bovine submaxillary mucoprotein before (————) and after (— — — —) treatment with RDE.

(A) Treatment with 20,000 units of RDE for 1 hour at 37° C. in calcium acetate (pH 6·0, μ=0·05).

(B) 50,000 units of RDE for 3 hours at 37° C. in calcium acetate (pH 6·0, μ=0·05).

The vertical lines indicate the initial positions of the boundaries and the arrows the direction of migration. The upper pattern represents the ascending limb.

Action of RDE. Pye (1955) has established that RDE reduces the net negative charge of the inhibitors of haemagglutination and that this reduction may be used as an assay for the presence of the influenza virus substrate. An 0·4 p.c. (w/v) solution of the mucin was treated with 20,000 units of RDE in calcium acetate buffer (pH 6·0, μ=0·05) for one hour at 37° C. Electrophoresis in phosphate buffer (pH 6·9, μ=0·2) showed that the mobility had been reduced to $-4{\cdot}5\times10^{-5}$ cm^2. sec^{-1}. volt^{-1} (Fig. 1a), all of the material migrating as a single peak. It was thought that more extensive treatment with RDE might reduce the mobility further and, consequently, another sample of the mucin was incubated with 50,000 units of RDE for three hours, all other conditions remaining as before. The mobility of this sample was reduced to $-2{\cdot}9\times10^{-5}$ cm^2. sec^{-1}. volt^{-1} (Fig. 1b), a reduction comparable to, or greater than that found with other inhibitory mucins. A number of samples which gave a single peak in phosphate buffer (pH 6·9, μ=0·2) appeared heterogeneous in the same buffer after RDE treatment although the mobility of the material was reduced (Fig. 2). This effect has not been observed with other inhibitory mucins (Pye, 1955) and it was not possible to resolve the anomalous preparations by further fractionation.

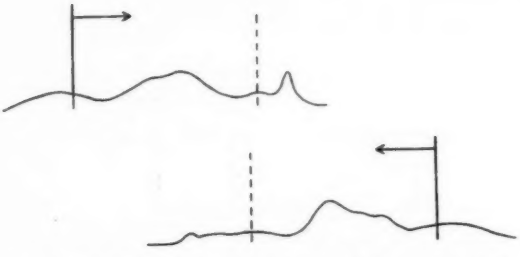

Fig. 2. Electrophoretic pattern showing marked heterogeneity of one preparation after RDE treatment. Electrophoresis was carried out in phosphate (pH 7, μ=0·2). The vertical broken line indicates the position which would be occupied by the single peak of the untreated preparation after electrophoresis under the same conditions.

Biological properties of B.S.M. inhibitor.

The inhibitory activity of the purified mucin was investigated using a wide range of viruses both as active and indicator preparations. The following viruses were tested against an 0·4 p.c. (w/v) solution; MEL, WSE, PR8, Ocean Island, BEL, LEE, BON, MIL.B, Taylor's 1233, NDV and Swine. The results are given in Table 1.

It can be seen that only the PR8 and 1233 viruses are inhibited to any extent. PR8 is inhibited only in the indicator state whilst heated and active

TABLE 1.

The inhibitory activity of a 0·4 p.c. solution of bovine submaxillary mucin towards different strains of influenza virus.

Virus	MEL	WSE	PR8	BEL	Ocean Island	LEE	BON	MIL. B	1233	NDV	Swine
Heated virus	10	80	24,000	10	10	320	20	10	1,000	10	10
"Active" virus	10	10	50	10	10	±10	10	10	1,400	10	10

1233 are inhibited. The inhibition of active 1233 is an unreliable result insofar as it was found extremely difficult to make enzymically active preparations of this strain. LEE and WSE are the only other viruses towards which even a weak activity is shown. In all cases the inhibitory activity was destroyed by RDE.

Rate of destruction of inhibitory activity to PR8 by RDE. In view of the extensive RDE treatment necessary to bring about a significant mobility reduction in the mucin, experiments were carried out to see whether such treatment was necessary to destroy inhibition to PR8 virus. This was done by comparing the rate of destruction of the inhibitory activity of the bovine mucin with that of the purified urinary inhibitor of Tamm and Horsfall (1952). It was found that the two reactions had similar velocity constants indicating that RDE destroys the inhibitory activity of BSM much faster than it reduces its mobility.

Enzymic action of viruses. In these experiments 30,000 agglutinating doses of purified MEL, WSE, LEE and PR8 viruses were allowed to act on 15 ml. samples of an 0·4 p.c. (w/v) solution of the mucin in calcium borate buffered saline for 3 hours at 37° C. The mixtures were then heated at 65° C. for 30 minutes to destroy virus and titrated against "indicator" WSE, LEE and PR8. In Table 2 the residual inhibitor activity in each of these titrations is expressed as a percentage of the inhibitor in a control to which boiled virus had been added.

TABLE 2.

The enzymic action of viruses on bovine submaxillary mucin.

Indicator	WSE	MEL	LEE	PR8
T.WSE	10	100	5	5
T.LEE	20	100	5	5
T.PR8	60	100	0·5	0·5

All figures represent residual titres expressed as percentages of the titres of control solutions which had been incubated with heat inactivated virus.

The results show that only PR8 and LEE viruses have any extensive action on the mucin. It is of interest that LEE, although giving a low inhibitory titre, is capable of destroying the inhibitory activity of the mucin for PR8.

Effect of virus action on the electrophoretic mobility. The mucin samples treated with virus in the previous experiment were submitted to electrophoresis in the phosphate buffer (pH 6·9, μ=0·2). The mobility values are given in Table 3.

TABLE 3.

Electrophoretic mobility of bovine submaxillary mucin after the action of different viruses. (Measured in pH 6·9 ionic strength = 0·2 potassium phosphate buffer.)

Mobilities expressed in 10^{-5} *cm.*^{2} *sec.*$^{-1}$ *volt*$^{-1}$

Virus	Mobility	Virus	Mobility
MEL	5·1	LEE	5·0
WSE	5·3	PR8	4·9
Control	5·4—5·0		

It can be seen that no virus preparation has caused a significant reduction in the mobility, an unexpected result since studies on other mucins had indicated that destruction of inhibitory activity by the virus enzyme was paralleled by a reduction in mobility. Exhaustive treatment with large amounts of PR8 virus (100,000 units for 24 hours) also failed to reduce the mobility. This suggested that partial treatment with RDE might destroy the inhibitory activity to PR8 virus without reducing the mobility. The reaction rate experiments had shown that treatment at 37° C. for thirty minutes with 10,000 units/ml. of RDE in calcium acetate buffer (pH 6·0, μ=0·05) was just sufficient to destroy the inhibitory activity of the mucin towards indicator PR8. These samples were submitted to electrophoresis in phosphate buffer (pH 6·9, μ=0·2) and it was found that the mobility was not reduced.

Purification of an inhibitor from sheep submaxillary glands.

Since barium-methanol fractionation led to the isolation of an inhibitory component from the bovine submaxillary gland extracts it was considered that the method might be applicable to the fractionation of sheep gland extracts. Sheep submaxillary glands were fractionated in exactly the same manner as described for the bovine glands. Electrophoretic examination revealed one component in phosphate buffer (pH 6·9, μ=0·2) with a mobility of $-5{\cdot}6\times10^{-5}$ cm^2. sec^{-1}. volt^{-1}. After treatment with RDE (0·4 p.c. solution of inhibitor, 5,000 units of RDE/ml. in calcium acetate buffer (pH 6·0, μ=0·05) at 37° C. for thirty minutes) this mobility was reduced to $-3{\cdot}0\times10^{-5}$ cm^2. sec^{-1}. volt^{-1}. This mobility is similar to that obtained with the bovine mucin after much more extensive RDE treatment. The purified inhibitor had the same biological properties as that present in crude extracts of the gland (Burnet, 1949) and as isolated by McCrea (1953).

DISCUSSION.

The present work is of special interest in relation to the concept of the receptor gradient for red cells (Burnet, McCrea and Stone, 1946) and the inhibitor gradient for soluble mucoprotein inhibitors (Stone, 1949b). Both are based on the fact that when a mucoprotein is acted on enzymically by an influenza virus it will lose its capacity to react with that virus but may retain reactivity toward another strain of virus. B.S.M. in its natural state is unable to inhibit indicator virus MEL and is insusceptible to its enzymic action, it has only a trivial inhibitory effect on indicator WSE and a slightly greater one on indicator LEE. In accordance with the general rule the enzymic action of WSE is slight. LEE, however, is as active enzymically as PR8, the only strain which is typically inhibited in the indicator phase.

By using electrophoretic measurements in parallel with biological tests for inhibitory action, a direct approach is possible to the hypothesis of "accessibility" of receptors which has been evoked (e.g., by Burnet, 1951) to explain the existence of gradients. Action by PR8 or LEE results in the destruction of inhibitory action without detectable change in electrophoretic mobility. Similarly brief action by the soluble enzyme RDE removes the inhibitory property without electrophoretic change. Prolonged action by RDE however, produces a striking reduction in electrophoretic mobility, presumably by destruction of receptor groups accessible only to the smaller molecule of RDE.

These findings are in general accord with the work of Stone (1949b) on inhibitor gradients which clearly indicated the existence of receptor groups of varying susceptibility or accessibility to different viruses on soluble inhibitors. Gottschalk's (1952, 1954) work on urinary inhibitor gave rise to the concept of the inhibitor mucoprotein as a protein macromolecule conjugated with a large number (about 150 in the urinary mucoprotein) of individual prosthetic groups. These groups are intimately involved in the receptor mechanism and may be responsible for all the biological activities ascribed to "receptors." The data obtained in the present work, however, would be in accord with any type of receptor which, on inactivation by the appropriate enzyme, showed a nett gain in positive charge. The essential point that emerges is that the groups concerned are so disposed in relation to other structures that only a small proportion is accessible to virus and only one type of virus, PR8, is effectively bound when in the indicator state. The small number of accessible groups will hold enzymically active LEE virus firmly enough for its action on these, but they cannot attach to indicator virus sufficiently firmly to compete with red cell receptors and thus act as an effective inhibitor. The existence of a gradient of the type found in these reactions probably in itself implies that substrate groupings vary in their accessibility, but the demonstration in the present work that RDE first attacks the same groupings as react with the viruses makes B.S.M. the first example in which the difference in accessibility can be shown directly.

SUMMARY.

An extract of bovine submaxillary glands has been fractionated by methanol in the presence of barium to yield a preparation exhibiting a single peak when submitted to electrophoresis in a phosphate buffer (pH 7·0, μ=0·2).

This preparation has been shown to inhibit to a high titre PR8 strain of influenza virus, to a low titre LEE and 1233 strains, and to be inactive towards eight other strains of haemagglutinating virus.

Destruction of the inhibitory activity by PR8, LEE or by the receptor destroying enzyme of *V. cholerae* (RDE) does not cause a significant change in the electrophoretic mobility. However, extended treatment with large amounts of RDE reduces the mobility from $-5{\cdot}4\times10^{-5}$ to $-2{\cdot}9\times10^{-5}$ cm^2. sec^{-1}. volt^{-1}.

These effects have been explained on the basis of the hypothesis of varying accessibility of receptors.

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ASSAY OF INHIBITORS OF INFLUENZA VIRUS HAEMAGGLUTINATION BY ELECTROPHORESIS

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It has been shown (Hanig, 1948; Ada and Stone, 1950) that influenza viruses and the receptor destroying enzyme (RDE) of *Vibrio cholerae* when allowed to act on erythrocytes, reduce their electrophoretic mobility. As it seemed likely that the action of enzymically active virus and RDE in destroying the power of certain mucoproteins to inhibit haemagglutination is similar to their action in rendering red cells inagglutinable by virus (Burnet, 1951), one might expect the action of influenza virus or RDE to alter the electrophoretic properties of the soluble inhibitors. If this is so, then electrophoresis of the normal and destroyed inhibitors should reveal a difference due to a change in mobility of the inhibitory component.

In the last few years, inhibitors from numerous sources have been described and the electrophoretic behaviour of several has been investigated in this laboratory. In every case it has been found that a difference in the electrophoretic pattern occurs following treatment with virus or RDE. Invariably the mobility toward the anode of part or appreciably all of the preparation has been reduced.

These experiments suggested a method of assay for the inhibitor content of a preparation. Such a test would be based on the principle that if one superimposed the patterns obtained before and after virus or RDE treatment, making the initial boundaries coincide and correcting for different values of concentration, field strength and time of electrophoresis, then by subtracting the curves it should be possible to estimate the fraction of the total material which has its mobility reduced.

Two inhibitors have been investigated along these lines and the results published (Curtain, French and Pye, 1953; Marmion, Curtain and Pye, 1953). It is the purpose of this paper to compare the results obtained with these and other inhibitors.

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Materials and Methods.

Virus and RDE.

LEE and MEL strains of influenza virus were purified from infected allantoic fluids by adsorption on to and elution from red cells followed by high speed centrifugation.

Receptor destroying enzyme of *Vibrio cholerae* (RDE) was prepared by the method of Ada and French (1950).

Titrations of inhibitory activity were performed according to the method of Burnet (1948).

Treatment with virus or RDE. The inhibitor preparation was incubated at 37° C. with sufficient virus or RDE to reduce the inhibitory titre to less than 1 p.c. of the original value in one to two hours. Usually a few drops of toluene were added as preservative.

Buffers. The composition, pH, ionic strength (μ) and conductivity (κ) of each buffer used are shown in Table 1.

TABLE 1.

Composition	pH	μ	κ
0·15M HAc, 0·1M NaOH,	4·9	0·1	0·0040
0·053M HAc, 0·05M NaOH,	5·9	0·05	0·0020
0·084M KH_2PO_4, 0·075M KOH,	5·9	0·1	0·0047
0·091M KH_2PO_4, 0·0545M KOH,	6·9	0·2	0·0073
0·0455M KH_2PO_4, 0·0273M KOH,	7·0	0·1	0·0040
0·0227M KH_2PO_4, 0·0136M KOH,	7·0	0·05	0·0021
0·0357M KH_2PO_4, 0·0321M KOH,	7·9	0·1	0·0040
0·0127M Barbital, 0·01M KOH, 0·04M KCl	8·2	0·05	0·0036
0·0103M Barbital, 0·01M KOH, 0·04M KCl	8·8	0·05	0·0035
0·1M KOH,			0·0053
0·155M Glycine,	9·8	0·1	
0·1M NaOH,			0·0040
0·015M Glycine, 0·01M KOH, 0·04M KCl	9·5	0·05	0·0036

Composition, pH, ionic strength (μ) and conductivity (κ) of the buffers used in electrophoresis of the inhibitors and of egg albumin.

Electrophoresis was carried out at 2° C. in a Tiselius apparatus with Philpot-Svensson optical system (Holden, Ada and Pye, 1952). Cells used for analytical work were the standard 11 c.c. cell with long centre section (channel length 9·2 cm., cross section 0·75 cm^2.) and the 2 c.c. cell (channel length 5 cm., cross section 0·30 cm^2.). Preparative work was carried out in two ways: 1. Using an 11 c.c. cell with long centre section and open electrode vessels, boundaries were formed (Kahn and Polson, 1947) and fractions removed by the use of fine glass capillaries inserted into the cell from above; 2. Using an 11 c.c. cell with two short centre sections (Tiselius, 1937) in which fractions were isolated by displacement of the upper centre section.

Patterns were photographed (using the 5460 A line of the mercury spectrum) on Kodak Orthochromatic plates. The photographs were enlarged optically (linear magnification ×5) and traced manually. Areas under curves were determined using a planimeter. Accurate mobilities were determined only in the case of the urinary inhibitor and the egg albumin. In the case of the urinary inhibitor the boundaries were sufficiently sharp to be located by inspection. With egg albumin, centroidal ordinates were determined by counting squares.

To estimate the inhibitor content, the ascending and descending patterns of the normal material were traced on to squared paper as above. Those for the treated material were

traced independently with their magnification adjusted to allow for differing field strength, current, time and conductivity. The anomalous boundaries were subtracted from the curves and the remaining areas measured. Then the following adjustment of areas was carried out independently for the ascending and descending boundary systems. The ordinates of the treated patterns were adjusted by a factor equal to the ratio of the areas of the appropriate normal and treated patterns. These adjusted ordinates were plotted on the normal patterns, the origin being chosen so that the initial boundaries coincided. Measurement of the appropriate areas then gave an estimate of the inhibitor assay of the material.

EXPERIMENTAL.

Cyst mucin.

The first inhibitor investigated by electrophoresis in this laboratory was isolated from an ovarian cyst (cyst No. 38, French, Gunter and Motteram, 1951) by Dr. E. L. French according to the method of McCrea (see Burnet, 1948). The material, which had been stored in the dried state, was a white powder, readily soluble in the phosphate buffers to give a colourless, faintly opalescent and slightly viscous solution.

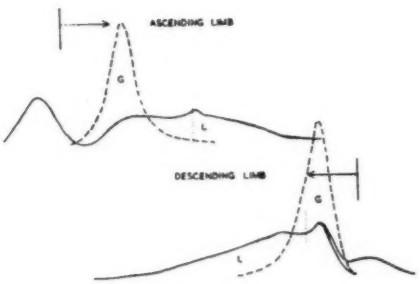


Fig. 1.

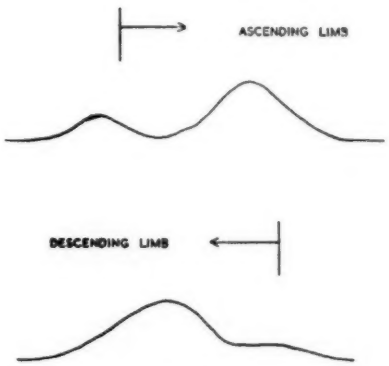


Fig. 2.

Fig. 1. Electrophoresis of cyst mucin before and after treatment with RDE. 4·7 volt cm^{-1}. for 16,800 sec. in phosphate buffer (pH 7·9 μ=0·1). The solid and broken lines indicate the patterns of the normal and RDE-treated material respectively. The vertical lines and arrows indicate the initial positions of the boundaries and the directions of migration.

Fractionation of the original material into fast and slow components was effected by inserting pipettes into the electrophoresis cell at the positions indicated on the pattern by the vertical dotted lines.

The areas L and G enclosed between the patterns of normal and RDE-treated inhibitor, excluding the anomalous boundaries, represent the loss of fast component and the gain in slow component, respectively, following the action of RDE. See also Table 3.

Fig. 2. Electrophoresis of electrophoretically purified cyst mucin at 5·7 volt cm^{-1}. for 7,800 sec. in phosphate buffer (pH 5·9 μ=0·2).

Fig. 1 shows the patterns obtained when the material was submitted to electrophoresis in phosphate buffer at pH 7·9 ionic strength (μ)=0·1, both before and after treatment with RDE. While the original material exhibited a very wide range of electrophoretic mobilities, it is seen that RDE treatment resulted in a reduction in the mobility of the faster components. The pattern of the treated material shows a much smaller range of mobilities. Similar results were obtained in phosphate pH 7·0 μ=0·1.

Assuming that RDE reduces the mobility of the inhibitory components, these results indicate that these components are the ones of higher mobility in the original preparation. In order to test this hypothesis, the material was submitted to fractionation in phosphate pH 7·9 μ=0·1, using pipettes to withdraw samples of the fast (A) and slow (B) components from the cell as shown in Fig. 1, where the dotted lines indicate the points at which pipettes were inserted. The samples recovered were titrated for inhibitory activity and known volumes were dried from the frozen state and weighed. After correcting for the mass of buffer salt present, the inhibitory activity per milligram dry weight of each sample and of the original material (C) was calculated. The results,

TABLE 2.

Run	Specific Activities		
	Fast Fraction A	Slow Fraction B	Control
1	38,000	1,800	—
2	97,000	4,800	35,000
3	48,000	2,400	30,000
4	33,000	500	20,000
5	33,000	4,400	20,000

Specific activities (expressed as inhibitory units per mg. dry weight) of the fast and slow fractions isolated from cyst mucin by electrophoresis.

which are shown in Table 2, indicate that the faster components are indeed more active as inhibitors than the slower ones.

On this basis the amount of inhibitor present in the material was calculated as outlined previously. The results, given in Table 3, led to the conclusion that the cyst preparation was at least 60 p.c. inhibitor.

Several of the fractions (A) were pooled and submitted to electrophoresis in phosphate pH 5·9 μ=0·2 (Fig. 2). The material, as expected, still appeared to be heterogeneous. This sample was required for other purposes and so was not treated with RDE.

TABLE 3.

(*a*) *Cyst Mucin.*
Ascending pattern.

	Loss of fast Components L	Gain in slow Components G	Total protein
Area	246	245	374
per cent of total protein	66	66	

Descending pattern.

Area	274	280	488
per cent of total protein	56	57	

(*b*) *Meconium Inhibitor.*
Ascending pattern.

Area	21	18	85
per cent of total protein	25	21	

Descending pattern.

Area	31	31	97
per cent of total protein	32	32	

Assay of (*a*) cyst mucin and (*b*) meconium inhibitor by superposition of patterns obtained before and after RDE treatment. Areas are given in arbitrary units. Total protein areas are the total areas of the patterns excluding the anomalous boundaries. The areas L and G are those shown in Figs. 1 and 3.

Meconium inhibitor.

The presence of an inhibitor in and its purification from human meconium has been described (Curtain, French and Pye, 1953). A preliminary description of its electrophoretic behaviour in phosphate pH 6·9 μ=0·2 was given in this report.

As in the case of cyst mucin, the material was heterogeneous and a large fraction of the fast components had its mobility reduced by RDE treatment. Fractionation in the cell with two centre sections once again showed that the faster components contained the bulk of the inhibitory activity. Typical patterns for normal and RDE treated meconium inhibitor are shown in Fig. 3. Calculations indicated that about 25–30 p.c. of the preparation was inhibitor (Table 3).

Urinary inhibitor.

In 1951, Tamm and Horsfall described the presence of an inhibitor in human urine. Several methods for its purification were given, but it appeared fairly clear that its insolubility in salt solution would lead to a mild method of isolation. Furthermore, it seemed that large amounts would be readily obtainable in a reasonably pure state due to the low protein content of urine. In a later publication (Tamm and Horsfall, 1952) full details of a method of purification based upon its insolubility in 3 p.c. salt solution were given. In addition, an inactive fraction was separated from their preparation due to a differential solubility in phosphate buffer pH 6·8 μ=0·05.

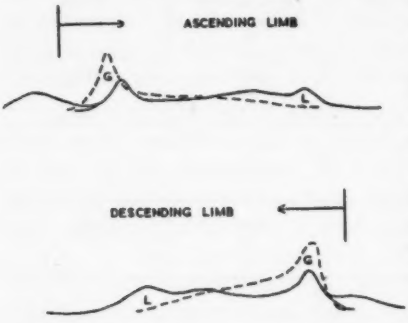


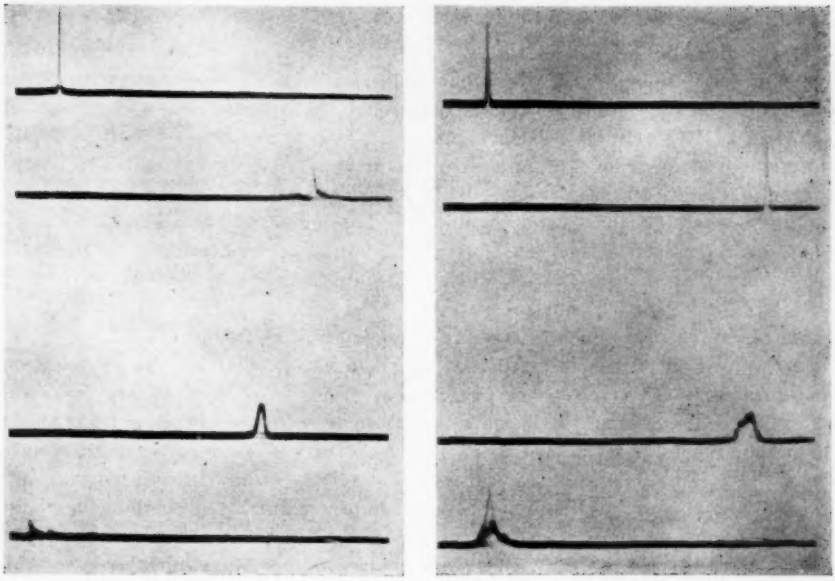
Fig. 3. Electrophoresis of normal and RDE-treated meconium inhibitor at 3·6 volt cm^{-1}. for 10,680 sec. in phosphate buffer (pH 6·9 μ=0·2). L and G bear the same significance as they do in Fig. 1.

In an accompanying paper Perlmann, Tamm and Horsfall (1952) described the electrophoretic behaviour of their material. Using a phosphate buffer (pH 6·8 μ=0·05) and a potential gradient of 6 volt cm^{-1}., the preparations were considered to be essentially homogeneous, although there was a tendency to obtain "saw-tooth" types of patterns. Treatment with LEE virus reduced the mobility of all the material from —8·4 to —6·7×10^{-5} cm^2. sec^{-1}. volt^{-1}. Electrophoresis of mixtures of equal amounts of treated and untreated inhibitors gave two peaks with the expected mobilities.

This was the first report of an inhibitor which appeared to be pure by physical criteria. Consequently it promised to be suitable for exhaustive chemical and physical investigation.

Several papers on its physical properties have been published (Curtain, 1953a, b, c) and details of electrophoretic studies are given here. The material used was prepared by methods which differed slightly from those reported by Tamm and Horsfall (Ada and Gottschalk, 1952; Curtain, 1953a).

Preliminary experiments showed that in phosphate buffer (pH 7·0) optimum results were obtained when μ=0·05. A field strength of 4 volt cm^{-1}. was used. Under these conditions, and providing the initial boundaries were carefully moved into the cell, a single peak was usually obtained in the ascending limb and occasionally in the descending limb. More frequently, however, the descending boundary was destroyed because the inhibitor solution did not drain sufficiently rapidly from the walls of the cell (see also Perlmann, Tamm and Horsfall, 1952). Thus all mobilities were calculated from the ascending limb.



(A) (B)

Fig. 4. Electrophoresis of two samples of urinary inhibitor (0·2 p.c. solution in phosphate buffer, pH 7·0 μ=0·05).

Boundaries are shown both before and after electrophoresis to illustrate the difficulties of obtaining satisfactory boundaries in the descending limb (lower pattern in each case).

A. Field strength 3·7 volt cm^{-1}. for 13,800 sec.

B. Field strength 3·7 volt cm^{-1}. for 17,460 sec. Note the splitting of the ascending boundary in this case.

Fig. 4 (a) shows a typical pattern obtained by electrophoresis of the inhibitor under the above conditions. The material has moved as one sharp peak (mobility $-9{\cdot}1\times10^{-5}$ cm^2. sec^{-1}. $volt^{-1}$) under a potential gradient of 3·7 volt cm^{-1}. for 13,800 sec. This potential gradient is less than that used by Perlmann *et al.* which was 6 volt cm^{-1}. Sometimes the peak began to split into two or three peaks (Fig. 4b). It is uncertain whether this was due to electrophoretic inhomogeneity, to effects of the high viscosity and low concentration (and hence low density increment) of the solutions (see Perlmann, Tamm and Horsfall, 1952), or to batch variation. Experiments with concentrations ranging from 0·05 to 0·2 p.c. failed to reveal any correlation with concentration. Comparable results were obtained at pH values ranging from 5·9 to 9·5. The inhibitor became more insoluble at pH values below 6.

Treatment of urinary inhibitor by virus and RDE.

Curtain (1953a) has shown that after treatment of urinary inhibitor with virus or RDE there was a slight but definite increase in viscosity at an ionic strength of 0·05. With exhaustive treatment the material often came out of solution, particularly after RDE treatment. Thus it has been difficult to obtain satisfactory preparations of treated inhibitor and virtually impossible to obtain a true end-point of the reaction. This probably accounts for the variations in mobility found for the treated inhibitors. The lowest values obtained for both RDE and virus (LEE and MEL) treated inhibitors were about $-6{\cdot}5\times10^{-5}$ cm^2. sec^{-1}. volt^{-1} (compared with mobilities ranging from $-8{\cdot}3\times10^{-5}$ to $-9{\cdot}1\times10^{-5}$ cm^2. sec^{-1}. volt^{-1} for the normal material). As with the normal inhibitor, single peaks were obtained which often showed evidence of splitting.

Application of the test outlined above indicated that all the preparation had inhibitory activity. Since the concentrations used were so low however, it would be impossible to detect a contaminant contributing less than about 10 p.c. of the total refractive index increment unless the peak were extremely sharp. Further, it is possible that interaction may occur between normal and treated components. Although the presence of an untreated component would lead to the formation of a fast moving boundary, the effect of interaction would be to decrease the concentration of this free component moving in front of the boundary corresponding to the treated component. This effect would further decrease the sensitivity of the method for detecting contaminants or untreated inhibitor.

Whether interaction can occur between normal and treated inhibitors can be tested by submitting mixtures of these materials to electrophoresis. Interaction would then result in deviations of the areas of the peaks and of their mobilities from those expected if interaction were absent. Because of the low concentrations used, calculation of areas under the peaks is insensitive. However, the mobilities may be determined fairly accurately. Table 4 shows the results

TABLE 4.

Description	Mobilities			
	Normal	Treated	Mixture	
1. LEE Treatment	8·7	6·8	8·5	7·6
2. LEE Treatment	8·4	7·3	8·5	8·1
3. RDE Treatment	8·7	6·5	8·9	7·9
4. RDE Treatment	8·3	6·5	8·4	7·6
5. RDE Treatment	8·3*	6·2*	7·9*	6·2*

* Electrophoresis conducted in the presence of M/200 ethylene-diamine-tetra-acetic acid (disodium salt).

Mobilities of the two components shown on electrophoresis of mixtures of equal proportions of normal and virus or RDE treated urinary inhibitors.

of typical experiments with mixtures containing equal amounts of normal and treated preparations. It is seen that in the first four cases the mobility of the normal component in the mixture is unchanged, whereas that of the treated component is considerably increased independently of whether virus or RDE treatment is involved.

Curtain (1953a) has shown that the presence of the divalent cations of calcium, magnesium and barium has a marked influence on the viscosity and solubility of the urinary inhibitor. It was considered possible, therefore, that the presence in particular of calcium ions might play a large part in the mechanism of interaction between normal and treated urinary inhibitors. Attempts were made to reduce this interaction by prolonged dialysis against distilled water of both the normal and treated inhibitors. This seemed to have no effect on the patterns obtained. Therefore experiments were carried out in the presence of the disodium salt of ethylene-diamine-tetra-acetic acid (EDTA) which binds divalent cations strongly.

M/2,000 EDTA was found to have a slight effect on the mobility of the slow component. The Table shows the result of an experiment using M/200 EDTA. Here the treated component shows the "correct" mobility while that for the normal is a little low, but probably still within experimental error.

Sputum inhibitor.

The preparation and electrophoretic properties of this material have been described previously (Marmion, Curtain and Pye, 1953). It was shown that a single peak was obtained in both phosphate (pH 7·0 μ=0·1) and glycine (pH 9·8 μ=0·1) buffers (Fig. 5a, 5b). The mobilities were $-6{\cdot}9\times10^{-5}$ and $-6{\cdot}0\times10^{-5}$ cm^2. sec^{-1}. volt^{-1} respectively. After RDE treatment single peaks were still obtained (Fig. 5a, 5b), the mobilities being reduced to $-5{\cdot}0\times10^{-5}$ and

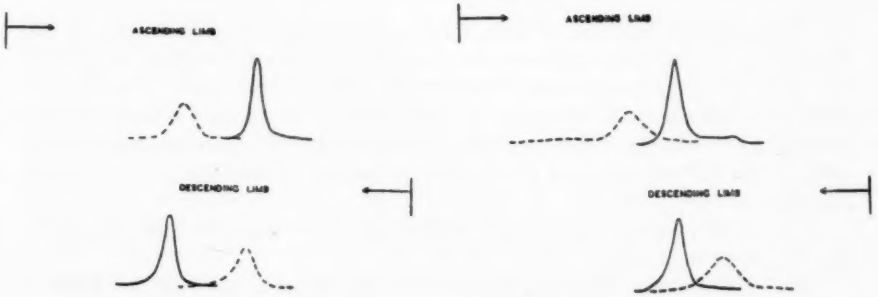


Fig. 5. Electrophoresis of sputum inhibitor before and after RDE treatment.
A. 3·4 volt cm^{-1}. for 14,820 sec. in phosphate buffer (pH 7·0 μ=0·1).
B. 2·5 volt cm^{-1}. for 20,160 sec. in glycine buffer (pH 9·8 μ=0·1).

$-4 \cdot 5 \times 10^{-5}$ cm^2. sec^{-1}. volt^{-1}. Application of the above method of assay indicated that all of the material was inhibitor. Unfortunately the material was so turbid that experiments on mixtures of normal and RDE treated material were impossible.

It should be noted that on many occasions skew boundaries or traces of other components were observed. This is to be expected because the preparation is derived from pathological material and so would be subject to variation between the individual samples of sputum included in each batch.

Egg albumin.

It has been shown that the electrophoretic properties of all of the inhibitors studied are modified by treatment with RDE. The possibility remains that this property is general to all proteins. An indication that this is not so is seen in the fact that appreciable fractions of cyst mucin and of meconium inhibitor appear to be unaffected by RDE. However, it seemed wise to submit to electrophoresis a protein with no inhibitory activity. Crystalline egg albumin prepared according to the method of Hopkins (1900) was chosen for this purpose. Titration against T-PR8 virus indicated no inhibitory action towards haemagglutination. Two 15 ml. portions of a 3 p.c. (w/v) solution (one containing 500,000 units of purified RDE) were incubated in the presence of toluene at 37° C. for two hours, diluted to 1 p.c. and submitted to electrophoresis in acetate buffer (pH 4·9 μ=0·1) for 25,400 sec. at a field strength of 3·3 volt cm^{-1}. The patterns of the treated and untreated samples were of the same shape and the small difference in their mobilities would correspond to a change in pH of less than 0·1. This is within the experimental error, and so it was concluded that RDE had no effect on the electrophoretic mobility of egg albumin.

DISCUSSION.

The primary objective of this study was to monitor experiments on the purification of inhibitor substances by providing a means of assaying the proportion of material present which could be regarded as inhibitor. The method described depends on the difference in behaviour of the purified or semi-purified material on the one hand and the same material treated by semi-purified RDE or virus. This will only be valid if certain implicit assumptions are correct. These assumptions are:

(1) RDE preparations do not contain any other enzymes that may significantly affect the results.

(2) All material whose electrophoretic behaviour is modified by RDE is inhibitor. All that can be said in this connection is that it was the rule during the period of exploratory search for inhibitors of haemagglutination in this Institute to concentrate on materials which showed relatively high capacity to inhibit haemagglutination by indicator viruses. In all cases RDE inactivated

these inhibitors and those which have been examined by electrophoresis exhibit a decrease in mobility after RDE action.

(3) There is no interaction between active and RDE destroyed inhibitors or between either of these and other components of the system. This assumption could be partly justified if a mixture of normal and RDE treated inhibitors gave an electrophoretic pattern which was merely a superposition of those of the separate components. Urinary inhibitor was the only material suitable for such a test, and Perlmann, Tamm and Horsfall (1952) have claimed that such is the case with urinary inhibitor mixed with LEE treated material. However, we were unable to repeat their results, irrespective of whether virus or RDE was used. The RDE (or virus) treated component in the mixture always showed a higher mobility than when tested alone.

No explanation of the difference between our results and those of the American workers is available, but the "correct" results obtained in experiments in which EDTA was present throughout the system suggest that the concentration of divalent cations present may play an important part in this interaction. The unusual physical properties of urinary inhibitor may well be responsible for this anomaly, but it must introduce an element of uncertainty into the present method of assaying the inhibitor content of a mucoprotein preparation.

A further difficulty is introduced whenever the peaks corresponding to treated and untreated inhibitors overlap. If this occurs the figure obtained will be too low by an amount equivalent to the extent of overlapping. This situation occurs in the case of cyst mucin and meconium inhibitor. Here the original patterns reveal a wide range of mobilities which is considerably reduced after RDE treatment. Although the assays show 60 and 30 p.c. respectively, it is probable that the inhibitor content is greater. It is possible that some of the slow material has some inhibitory activity but undergoes a smaller change in mobility following RDE treatment. It may be that this is a product of partial degradation of more active material.

The problem of overlapping does not occur in the case of the other two inhibitors. In both cases the tests showed that within the sensitivity of the optical system the mobility of all of the material was reduced. On the evidence available it is impossible to say whether either inhibitor is strictly "electrophoretically homogeneous." Urinary inhibitor does not lend itself to study by the usual tests of homogenity (Alberty, 1948; Hoch, 1950) because of its physical properties. However, it moves as a substantially single peak at pH values between 6 and 10, although there is often evidence of splitting of the peak. Because of its turbidity sputum inhibitor also was unsuitable for testing in this way. Irrespective of physical criteria of purity however, both preparations can be regarded as being "pure" substrates for RDE, implying only that all the material is susceptible to RDE action.

SUMMARY.

Electrophoresis of some inhibitors of influenza virus haemagglutination is described. After destruction of the inhibitory activity with the receptor destroying enzyme of *Vibrio cholerae*, the electrophoretic patterns of the materials are changed in a manner consistent with a decrease in the net negative charge of some or all of the molecular species present. The action of LEE and MEL strains of influenza virus produces the same charge reduction with the urinary inhibitor. These findings are used as the basis for the assay of samples for their inhibitor content. It is believed that cyst mucin and meconium inhibitor contain respectively 60 and 30 p.c. inhibitor while urinary inhibitor and sputum inhibitor are substantially pure as inhibitors.

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THE DETERMINATION OF VITAMIN B_{12}-ACTIVITY IN THE ORGANS AND EXCRETA OF SHEEP

3. THE EXCRETION OF VITAMIN B_{12}-ACTIVE FACTORS IN THE FAECES AND URINE OF SHEEP

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In the earlier papers in this series the microbiological assay methods used by us for the determination of vitamin B_{12} and allied factors were described and discussed. We also presented data on the vitamin B_{12}-activity of the rumen contents of sheep confined in pens or roaming on pasture (Dawbarn and Hine, 1954; Hine and Dawbarn, 1954). The present paper embodies the results of a study of the excretion of vitamin B_{12}-activity by sheep under controlled conditions. It includes a comparison of cobalt-deficient animals with those receiving cobalt by mouth, vitamin B_{12} by mouth or vitamin B_{12} by injection.

All the animals were confined in metabolism cages and were subsisting on a cobalt-deficient diet of wheaten hay chaff containing 5 to 6 p.c. gluten. The chaff contained between 0·019 and 0·037 μg. cobalt per gm. dry wt. In all but two experiments a stabilization period preceded the period of 14 days during which daily aliquots of urine and faeces were collected. In two experiments adaptation to a change in treatment was studied by an examination of urine and faeces collected immediately before the treatment was begun and at intervals thereafter.

METHODS.

Faeces.

Prior to assay all samples were stored at —20° C. 10 gm. aliquots were homogenized in a Waring Blendor in 200 ml. glass distilled water containing 4 mg. HCN. The reaction was adjusted to pH 5·0 with HCl and the mixture was transferred to a ruby flask in a final volume of 250 ml. and steamed for 30 minutes in a boiling autoclave at atmospheric pressure. After cooling, the extracts were centrifuged and filtered and the reaction of the filtrates was brought to pH 6·8.

The vitamin B_{12}-activity in the extracts was determined by four microbiological methods, i.e., a plate assay using a mutant of *Escherichia coli* as the test organism and three tube assays with *E. coli* mutant, *Lactobacillus leichmannii 313* and *Ochromonas malhamensis*.

Since the publication of the preceding paper in this series it has been reported that, although *Ochromonas* does not respond to pseudo-vitamin B_{12}, factor A or factor B, there are other vitamin B_{12}-like factors which are active for this micro-organism (Ford, Holdsworth and Kon, 1954).

Urine.

Aliquots of the 24-hour urines were treated with cyanide (0·5 ml. 1 p.c. KCN per 100 ml. urine), the reaction was adjusted to pH 6·8 and the samples were boiled for half a minute. They were stored at —20° C. until the assays were carried out.

In this investigation the urines were not heated further before assay. In 5 tests a comparison was made of the vitamin B_{12}-activities of unheated urines and of urines diluted 5 or 10 times and steamed for 30 minutes at an initial pH of 5·0. In every case the activity was less after steaming. However, there appeared to be no loss of pure vitamin B_{12} added to urine before steaming (one experiment only). (Table 1.)

The vitamin B_{12}-activity of all urines was measured by the *L. leichmannii* method. In order to determine whether a multiplicity of active factors was excreted in the urine as in the faeces we attempted to carry out the assays by other microbiological methods. However, it was not usually possible to measure the activity in urine with *E. coli* mutant or *Ochromonas*. In the *E. coli* tube assay increasing concentrations of urine gave higher and higher estimates while with *Ochromonas* progressively lower measurements of activity were obtained. In the few instances where the vitamin B_{12} content of the urine was great enough to allow very considerable dilution before assay, i.e., about 100 times, satisfactory measurements could be made by all three methods (Table 3).

As a result of these findings we attempted to separate the vitamin B_{12}-active factors from the interfering material in the urines. The method adopted was based on certain steps in the concentration and isolation of vitamin B_{12} by Fantes, Page, Parker and Lester Smith (1949) and Ford and Porter (1953).

The reaction of the urine was adjusted to pH 5·5 with N.HCl and 50 ml. were shaken with 1 gm. Darco G60 for 30 minutes. The charcoal was filtered off, the filtrate discarded and vitamin B_{12}-activity eluted by heating the charcoal under reflux for 2 to 3 minutes with 100 ml. 65 p.c. acetone containing 0·004 p.c. HCN. The acetone was decanted off through filter paper and the residual charcoal in the flask again extracted with acetone. The combined acetone filtrates were concentrated under reduced pressure to remove the acetone, diluted to 50 ml. and the pH adjusted to 6·8.

Recoveries of the original vitamin B_{12}-activity after this treatment, as measured by the *L. leichmannii* method, are recorded in Tables 3 and 4.

Urines treated in this way could be assayed by the *Ochromonas* method if the activity permitted at least a 10-fold dilution (Table 2). However, in the *E. coli* tube method the slope of the dose response curve when urines were assayed was still greater than with vitamin B_{12}. The slope of the line when methionine was substituted for vitamin B_{12} as a growth factor for *E. coli*, was similar to that for vitamin B_{12}. It would seem that urines contain a factor which enhances the growth promoting capacity of the vitamin for this organism.

The proportion of the activity for *L. leichmannii* which was not destroyed by heating in alkali (usually attributed to nucleotides) was determined after diluting the urines 5-fold in a final concentration of N/10 NaOH and autoclaving for 15 minutes at 15 lb. pressure.

Ionophoresis.

It is apparent from the data presented in Table 3 that the greater part of the vitamin B_{12}-activity for *L. leichmannii* in the urines from 10 collection periods could be accounted for as vitamin B_{12} (estimated by *Ochromonas* assay) and alkali stable material. Other active factors could have been present only in traces, if at all.

In the urine excreted by sheep No. 0005, however, from 42 to 64 p.c. of the activity for *L. leichmannii* was due to factors not active for *Ochromonas* (Table 4).

A separation of the active factors was attempted by ionophoresis on paper (Holdsworth, 1953). The apparatus used was that described by Foster (1952). The solvent was 0·5 N

TABLE 1.

Effect of steaming on estimated vitamin B_{12}-activity in urine.

Urine No.	mμg. B_{12} activity per ml. urine (*L. leichmannii*)	
	Steamed	Not steamed
1	0·12	0·15
2	1·12	1·87
3	0·19	0·27
4	0·95	1·47
5	3·00	3·30
	+ 3·12 mμg. B_{12}/ml.	
	6·22	6·57

TABLE 2.

Effect of dilution on the estimated vitamin B_{12}-activity of urine: Ochromonas *assay.*

Sheep No.	Unpurified urine		Purified urine	
	Dilution	mμg. B_{12} per ml. urine	Dilution	mμg. B_{12} per ml. urine
9029	1 in 5	0·175	1 in 10	1·84
	1 in 10	0·64	1 in 17	2·10
	1 in 20	1·12	1 in 33	2·00
	1 in 40	1·52	1 in 67	1·87
	1 in 100	1·90	mean	1·95
0077			1 in 6	1·23
			1 in 13	2·14
			1 in 25	2·34
			1 in 50	2·22
0113			1 in 8	1·59
			1 in 16	3·07
			1 in 33	3·48
			1 in 66	2·95
7019	1 in 45	10·1	1 in 45	10·7
	1 in 67	10·8	1 in 67	10·6
	1 in 112	10·4	1 in 112	10·4
	1 in 225	11·2	1 in 225	10·3
7009	1 in 100	21·1	1 in 100	23·0
	1 in 150	23·4	1 in 150	23·4
	1 in 250	22·3	1 in 250	22·4
	1 in 500	24·3	1 in 500	23·3

TABLE 3.

Vitamin B_{12}-activity in the urine of sheep.

Sheep No.	Treatment	B_{12} in urine mμg./ml. (*L. leichmannii*) L	Activity not destroyed by alkaline autoclaving p.c. of L	B_{12} in purified urine (*L. leichmannii*) p.c. of L	B_{12}-activity estimated by other microbiological methods		
					Method	unpurified urine	purified urine
						p.c. of activity estimated by *L. leich.* (L)	p.c. of activity in purified urine estimated by *L. leich.*
5009 period F	cobalt deficient	0·30	44	97			
7019 period F	50 μg. B_{12} per day parenteral	11·9		85	*Ochromonas* *E. coli* tube *E. coli* plate	89 100 97	103 105 111
9151 period G	cobalt deficient	0·37	30	83			
9029 period G	1 mg. Co per day per os	1·94	8	88	*Ochromonas*		93
9090 period H	cobalt deficient	0·40	35	107			
7009 period H	50 μg. B_{12} per day parenteral	23·0		95	*Ochromonas* *E. coli* tube *E. coli* plate	100 97 97	106 111 100
0077 period J	cobalt deficient	0·35	32	89	*Ochromonas*		75
0113 period J	1 mg. Co per day per os	3·96	5	85	*Ochromonas*		94
0077 period K	1 mg. Co per day per os	2·38	5	95	*Ochromonas*		99
0113 period K	cobalt deficient	0·57	30	95	*Ochromonas*		69

TABLE 4.

Vitamin B_{12}-activity in the urine of sheep.

Sheep No.	Treatment	Day	B_{12} in urine mμg./ml. (*leichmannii*) L	Activity not destroyed by alk. autoclaving (p.c. of L.)	Purified urine	
					Activity estimated by *leich.* (p.c. of L.)	Activity estimated by *Ochromonas* p.c. of activity by *leich.*
5009	500 μg. B_{12} per day per os from day 0	−2 to 0	0·52	20	95	94
		0 to 1	0·62	25		
		1 to 2	0·68	19	86	64
		5 to 6	1·38		67	82
		12 to 13	1·11	14	80	69
		31 to 32	0·79	18	63	
0005	1 mg. Co per day per os on and before day 1 then cobalt withheld	1	8·5	3	57	36
		2	5·0	5	67	38
		3	4·9	5	76	45
		4	4·2	7	86	56
		5	3·6	10		
		6	2·7	12	71	47
		7	1·97	19		53
		8	1·91	18		
		9	1·78	16		
		10	1·25	21	76	58
		11	1·21			
		12	1·09			
		13	1·03			
		14	1·42	19	73	54

acetic acid containing 0·01 p.c. KCN. After ionophoresis with a potential difference of approximately 1,200 volts applied for 6–7 hours, the paper was dried in air at room temperature and cut into pieces each measuring 1 cm. along the direction of flow by 2·4 to 3 cm. wide. Each paper piece was placed in a tube containing 5 ml. medium and 5 ml. water, autoclaved and assayed in the usual way by the *L. leichmannii* method.

To obtain sufficient material for assay it was necessary to apply as much as 0·1 ml. urine to the paper. This was done with a micrometer syringe while a stream of cold air was directed on to the paper. The resulting spots were rather large, about 5 mm. across.

When untreated urine was examined a satisfactory separation of active factors was not effected, but with purified urine the presence of several factors was clearly shown (Fig. 1). True vitamin B_{12} appeared at a point 4 cm. from the origin, towards the cathode. This movement is believed to be due to electro-endosmosis. Another major centre of activity was found at —14 to —15 cm. and a lesser activity at —10 cm.

The total vitamin B_{12}-activity distributed throughout the paper was added and compared with that applied to the paper. A high degree of accuracy can hardly be expected from such a procedure. Nevertheless the recovery was reasonably good, i.e., 93 p.c., 84 p.c. and 107 p.c. for the urines of Day 2, Day 3 and Day 4, respectively. The proportion of the total activity due to true vitamin B_{12} and any factor not separated from it was 47 p.c., 62 p.c. and 53 p.c.

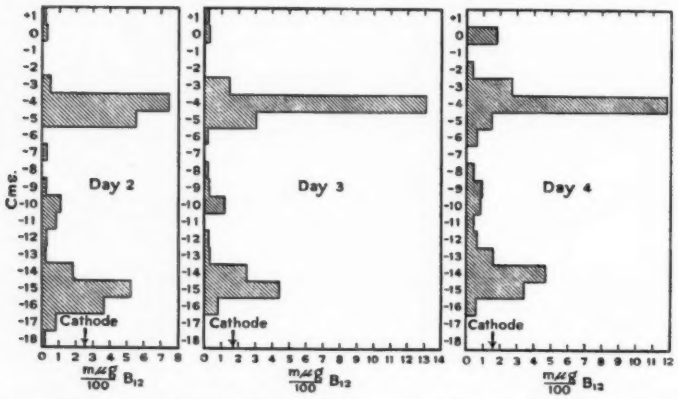

Fig. 1. Separation of vitamin B_{12}-active factors in urine by ionophoresis on paper.
Purified urine from Sheep No. 0005.
Days 2, 3 and 4.
Ionophoresis in 0·5 N acetic acid containing 0·01 p.c. KCN for 6 to 7 hours at about 1,200 V.
(*L. leichmannii* assay).

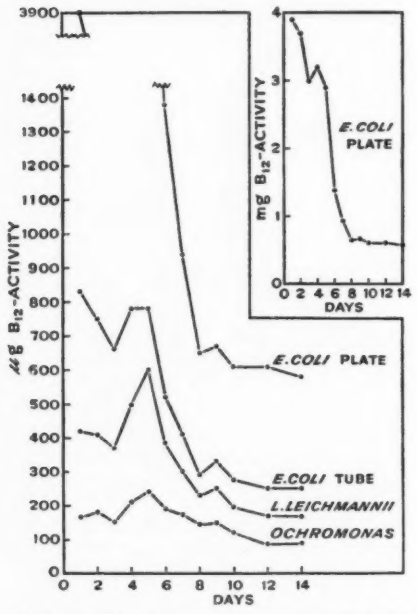

Fig. 2. The daily excretion of vitamin B_{12}-activity in the faeces of sheep No. 0005.
1 mg. Co per day *per os* on and before day 1, then Co withheld.

Fig. 3. The daily excretion of vitamin B_{12}-activity in the urine of sheep No. 0005.
1 mg. Co per day *per os* on and before day 1, then Co withheld.
(*L. leichmannii* assay, less activity stable to alkaline autoclaving.)

TABLE 5.

Vitamin B_{12}-activity in the faeces of sheep.

Collection period 14 days	Sheep No.	Treatment	Wet wt. faeces in period gm.	μg. B_{12}-activity per gm. dry wt.				μg. B_{12}-activity per 24 hr.				Ratio			
				E. coli plate a	*E. coli* tube b	*L. leich.* c	*Ochrom.* d	*E. coli* plate	*E. coli* tube	*L. leich.*	*Ochrom.*	a	b	c	d
E	9134	Co-deficient	2215	2·5	1·43	0·97	0·49	175	98	66	34	5·1	2·9	2·0	1
	7049	1 mg. Co per day per os, pair fed with 9134	3118	12·3	3·8	2·4	1·24	850	260	167	86	9·9	3·0	1·9	1
F	5009	Co-deficient	4820	1·5	0·72	0·47	0·168	190	91	59	21	9·1	4·3	2·8	1
	7019	50 μg. B_{12} per day parenteral, pair fed with 5009	4526	2·7	1·60	1·12	0·56	270	161	113	57	4·7	2·8	2·0	1
G	9151	Co-deficient	4606	1·4	0·65	0·42	0·122	210	98	64	18·4	11·2	5·3	3·5	1
	9029	1 mg. Co per day per os, pair fed with 9151	4351	12·9	3·4	2·0	0·83	1430	370	220	92	15·6	4·1	2·4	1
H	9090	Co-deficient	4853	1·6	0·96	0·64	0·26	260	163	108	44	6·0	3·7	2·5	1
	7009	50 μg. B_{12} per day parenteral, pair fed with 9090	5112	2·5	1·13	0·89	0·40	420	187	147	66	6·3	2·8	2·2	1
J	0077	Co-deficient	14660	2·2	0·80	0·76	0·40	690	250	240	127	5·4	2·0	1·9	1
	0113	1 mg. Co per day per os	14780	10·0	2·1	1·59	0·84	3100	670	490	260	11·9	2·6	1·9	1
K	0077	1 mg. Co per day per os	15130	10·5	2·3	1·79	0·94	3400	760	580	300	11·2	2·5	1·9	1
	0113	Co-deficient	14600	2·2	0·81	0·73	0·40	650	240	220	120	5·5	2·0	1·8	1

TABLE 6.

Vitamin B_{12}-activity in the faeces of Sheep No. 5009.

500 µg. B_{12} per day per os from Day 0.

Days after 1st dose of B_{12}		Wet wt. faeces in 24 hr. gm.	µg. B_{12}-activity per gm. dry wt.				µg. B_{12}-activity per 24 hr.				Ratio			
			E. coli plate a	*E. coli* tube b	*L. leich.* c	*Ochrom.* d	*E. coli* plate	*E. coli* tube	*L. leich.*	*Ochrom.*	a	b	c	d
−4 to −2	48 hr.	302	1·76	0·59	0·41	0·098	220	75	52	12·4	18	6·0	4·2	1
−2 to 0	48 hr.	102	1·21	0·45	0·29	0·089	63	23	15·1	4·6	14	5·0	3·3	1
0 to 1	24 hr.	127	1·63	0·66	0·40	0·126	76	31	18·8	5·8	13	5·2	3·2	1
1 to 2	24 hr.	352	3·6	1·27	0·90	0·42	490	174	123	57	8·6	3·0	2·1	1
5 to 6	48 hr.	290	5·6	2·40	1·67	1·15	590	260	177	121	4·9	2·1	1·5	1
12 to 13	48 hr.	463	3·5	1·63	1·11	0·63	620	290	199	112	5·6	2·6	1·8	1
31 to 32	48 hr.	670	2·1	1·24	0·86	0·54	510	290	202	126	4·0	2·3	1·6	1

TABLE 7.

Vitamin B_{12}-activity in the faeces of Sheep No. 0005.

1 mg Co per day per os on and before day 1, then cobalt withheld.

Diet: 800 *gm. wheaten hay-chaff and* 50 *gm. gluten daily.*

Day	μg. B_{12}-activity per gm. dry wt.				μg. B_{12}-activity per 24 hr.				Ratio			
	E. coli plate a	*E. coli* tube b	*L. leich.* c	*Ochrom.* d	*E. coli* plate	*E. coli* tube	*L. leich.*	*Ochrom.*	a	b	c	d
1	13	2·8	1·41	0·56	3900	830	420	166	23	5·0	2·5	1
2	12	2·5	1·38	0·60	3700	750	410	180	20	4·1	2·3	1
3	10	2·1	1·21	0·49	3000	660	370	151	20	4·4	2·5	1
4	10	2·4	1·51	0·63	3200	780	500	210	15	3·8	2·4	1
5	8·8	2·4	1·82	0·74	2900	780	600	240	12	3·2	2·5	1
6	4·3	1·63	1·21	0·60	1380	520	385	191	7·2	2·7	2·0	1
7	3·2	1·38	1·02	0·59	940	410	300	173	5·4	2·3	1·7	1
8	2·5	1·12	0·89	0·55	650	290	230	144	4·5	2·0	1·6	1
9	2·3	1·15	0·88	0·52	670	330	250	149	4·4	2·2	1·7	1
10	2·1	0·96	0·68	0·42	610	275	196	120	5·0	2·3	1·6	1
12	2·1	0·87	0·58	0·30	610	250	168	87	6·9	2·9	1·9	1
14	2·0	0·84	0·57	0·30	580	250	167	89	6·6	2·8	1·9	1

TABLE 8.

Vitamin B_{12}-activity in the urine of sheep.

Sheep No.	Treatment	Collection period 14 days	B_{12} in 24 hr. urine (*L. leichmannii* assay less alkali stable material)	
			μg. B_{12}	as p.c. of faecal excretion (*L. leich.*)
5009	Co-deficient	F	0·09	0·15
7019	50 μg. B_{12} per day parenteral, pair fed with 5009	F	15·2	13
9151	Co-deficient	G	0·07	0·11
9029	1 mg. Co per day per os, pair fed with 9151	G	0·76	0·35
9090	Co-deficient	H	0·11	0·10
7009	50 μg. B_{12} per day parenteral, pair fed with 9090	H	17·7	12
0077	Co-deficient	J	0·26	0·11
0113	1 mg. Co. per day per os	J	3·6	0·74
0077	1 mg. Co. per day per os	K	2·7	0·47
0113	Co-deficient	K	0·35	0·16

TABLE 9.

Vitamin B_{12}-activity in the urine of sheep.

Sheep No.	Treatment	Day	B_{12} in 24 hr. urine (L. leichm. assay less alkali stable material)	
			μg. B_{12}	as p.c. of faecal excretion (*L. leich.*)
5009	500 μg. B_{12} per day per os from day 0	−2 to 0	0·17	1·1
		0 to 1	0·09	0·5
		1 to 2	0·15	0·12
		5 to 6	0·27	0·15
		12 to 13	0·26	0·13
		31 to 32	0·18	0·09
0005	1 mg. Co per day per os on and before day 1, then Co withheld	1	1·89	0·45
		2	1·57	0·38
		3	1·85	0·50
		4	1·61	0·32
		5	1·22	0·20
		6	0·95	0·25
		7	0·53	0·18
		8	0·53	0·23
		9	0·55	0·22
		10	0·41	0·21
		11	0·43	
		12	0·34	0·20
		13	0·40	
		14	0·45	0·27

TABLE 10.

Vitamin B_{12}-activity in the faeces and rumen contents of sheep.

μg. B_{12}/gm. dry weight mean values.

Method of Assay

E. coli plate	*E. coli* tube	*L. leich.*	*Ochromonas*
Receiving 1 mg. Co per day per os *Faeces*—5 sheep (Tables 5 and 7)			
11·7	2·9	1·84	0·88
Rumen contents—4 collection periods (2 sheep)			
11·2	2·5	1·62	0·70
Cobalt-deficient *Faeces*—5 sheep (Tables 5 and 7)			
2·06	0·91	0·69	0·34
Rumen contents—4 collection periods (3 sheep)			
0·41	0·188	0·127	0·056

RESULTS AND DISCUSSION.

The results of these assays may be considered in three sections.

1. *The effect of administering cobalt* per os *to sheep subsisting on a cobalt-deficient diet.*

The excretion of vitamin B_{12}-activity was examined in four pairs of sheep. One of each pair received a daily drench of 1 mg. cobalt while the other received no supplementary cobalt. The results of these experiments are presented in Tables 5 and 8. In a further study cobalt was withheld from an animal which had been receiving it, and the effect on excretion was determined by assay of samples of faeces and urine collected daily for fourteen days (Tables 7 and 9).

The vitamin B_{12}-activity of the faeces of sheep receiving cobalt was markedly greater than that of cobalt-deficient sheep. This was shown by all four methods of assay and in all the animals studied. The estimated activity was invariably greatest when determined by the *E. coli* plate method and decreased step by step with the *E. coli* tube, *L. leichmannii* and *Ochromonas* methods. The ratio of activities as determined by the first and last of these methods had a mean

value of 14 to 1 in the faeces of sheep receiving cobalt and only 7 to 1 in cobalt-deficient sheep. It would seem that the proportion of active factors other than cyanocobalamin decreased in cobalt deficiency.

When sheep received adequate cobalt the concentration of vitamin B_{12}-activity in dry rumen contents and dry faeces did not differ appreciably, but when cobalt was lacking the activity in rumen contents was only about one-fifth that in faeces (Table 10).

In the urine the daily excretion of vitamin B_{12}-activity (*L. leichmannii* method, less material stable to alkaline autoclaving) varied, for cobalt-deficient sheep, from 0·09 to 0·45 μg. and for those receiving cobalt by mouth from 0·76 to 3·6 μg. When the cobalt drench administered to sheep No. 0005 was withheld the daily urinary excretion fell from 1·89 to 0·45 μg. in 14 days.

In all these instances the urinary excretion was less than 1 p.c. of the faecal excretion (Tables 8 and 9).

The proportion of alkali stable activity was much greater in the urines of cobalt-deficient sheep than in those of sheep receiving cobalt; the absolute amount excreted in the urine was affected little, if at all, by the level of cobalt ingested (Tables 3 and 4).

The effect of withholding the cobalt drench on the daily excretion of vitamin B_{12}-activity in faeces and urine is presented graphically in Figs. 2 and 3. It is apparent that equilibrium was reached in 12 days.

2. *The effect of giving vitamin B_{12} by mouth.*

When 500 μg. vitamin B_{12} were administered daily to a sheep the excretion in the urine was negligible, i.e., less than 0·3 μg. per 24 hours (Table 9).

The increase in the 24-hour faecal output of vitamin B_{12}-activity after 5 to 6 days was 450, 210, 145 and 115 μg. as measured by each of the four assay methods (Table 6). Less than a quarter of the ingested vitamin, therefore, was excreted unchanged. It is probable that part appeared in the faeces in other active forms, perhaps as factor B (denucleotided vitamin B_{12}; Gant, Lester Smith and Parker, 1954) or as other factors synthesized by the rumen or intestinal flora from factor B. Ford and Holdsworth (1954) observed that a mutant of *E. coli* growing in a medium containing factor B, synthesized factor A, pseudo-vitamin B_{12}, or cyanocobalamin, depending on the nucleotide supplied.

3. *The effect of administering vitamin B_{12} by injection.*

Two sheep received 50 μg. vitamin B_{12} per day by injection. Of this amount 15 μg. and 18 μg. appeared in the 24-hour urine of the two animals (Table 8).

The excretion of vitamin B_{12}-activity in the faeces of these sheep was compared with that of animals not receiving vitamin B_{12} with which they were pair-fed. The excess excretion was 36 μg. in one case and 22 μg. in the other, when measured by the *Ochromonas* method and more by the other methods of assay (Table 5).

Thus 30 to 36 p.c. of the injected vitamin was excreted in the urine, and part, possibly the greater part, of the remainder in the faeces.

SUMMARY.

The excretion of vitamin B_{12}-activity in the faeces and urine was studied in sheep subsisting on a cobalt-deficient diet of wheaten-hay chaff and gluten. A comparison was made of cobalt-deficient animals with those receiving cobalt by mouth, vitamin B_{12} by mouth or vitamin B_{12} by injection.

The vitamin B_{12}-activity in the faeces was measured by four microbiological assay methods; viz., an *E. coli* mutant plate assay and tube assays with *E. coli* mutant, *L. leichmannii* and *Ochromonas malhamensis* as the test organisms.

The urines were assayed by the *L. leichmannii* method and after purification by the *L. leichmannii* and *Ochromonas* methods.

The estimate of vitamin B_{12}-activity in the faeces was greatest when made by the *E. coli* plate method and decreased step by step with the other three methods. The activity, however it was measured, was much greater in sheep receiving adequate cobalt than in cobalt-deficient animals. The ratio of the activities as measured by the *E. coli* plate and *Ochromonas* methods was also greater in sheep receiving adequate cobalt. The decrease in vitamin B_{12}-activity in the faeces of cobalt-deficient animals as compared with normal sheep was much less than the corresponding decrease in the rumen contents.

The concentration of vitamin B_{12}-activity in the urine also reflected the cobalt status of the animal, although the daily excretion was less than 1 p.c. of the excretion in the faeces.

When the administration of a cobalt drench was discontinued there was a gradual decline in the vitamin B_{12}-activity in faeces and urine reaching equilibrium in 12 days.

A comparison of the *L. leichmannii* and *Ochromonas* assays of urines indicated the presence in the urine of one sheep of more than one vitamin B_{12}-active factor. An examination of the purified urine by ionophoresis showed clearly that two or more factors were present.

When 500 μg. vitamin B_{12} were given each day by mouth to a sheep on a cobalt-deficient diet not more than one quarter of the administered vitamin was excreted unchanged in the faeces. Less than 0·3 μg. appeared in the urine.

When 50 μg. vitamin B_{12} were injected each day 30 to 36 p.c. was excreted in the urine and a part, possibly the greater part, of the remainder in the faeces.

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THE EFFECT OF COPPER ON THE RESPONSE TO VITAMIN B_{12} AND METHIONINE OF A MUTANT STRAIN OF *ESCHERICHIA COLI*

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The requirements of a mutant of *Escherichia coli* for methionine can be met by vitamin B_{12} (Davis and Mingioli, 1950). These authors found that homocysteine was inactive for this purpose. Dubnoff (1952) reported that homocysteine, but not homocystine, promoted growth of the mutant under anaerobic conditions which enabled sulphydryl groups to be maintained in the reduced state. He concluded that vitamin B_{12} functions primarily in the reduction of homocystine to homocysteine. Gibson and Woods (1952) found, however, that washed suspensions of a vitamin B_{12}-requiring strain of *E. coli* failed to convert homocysteine to methionine until vitamin B_{12} was added. Shive (1953) suggested that the vitamin is involved in the methylation step of the biosynthesis of methionine by *E. coli*. It is clear that vitamin B_{12} functions in either or both of the steps of reduction and methylation through which the synthesis of methionine from homocystine proceeds.

A tube assay for vitamin B_{12} with an *E. coli* mutant as the test organism (Burkholder, 1951) is in use in this laboratory. Results which may have some bearing on methionine synthesis by *E. coli* were obtained when copper was added to the growth medium. The organism used in these experiments was an *Escherichia coli* mutant No. 8134 from the National Collection of Industrial Bacteria, D.S.I.R., England.

METHODS

The assay method resembled that of Burkholder (1951) except that thioglycollic acid was omitted from the basal medium and KCN (·001 p.c.) was added, together with appropriate amounts of the substances under test.

Solutions containing graded amounts of vitamin B_{12} or methionine were prepared and four replicates of each were dispensed, in a volume of 2·5 ml., into pyrex test tubes 16 mm. in internal diameter by 150 mm. in length; 2·5 ml. medium were added to each and the tubes were plugged with cotton wool. They were placed in a vigorously steaming autoclave and heated at 115° C. for 5 minutes. When the pressure returned to normal the tubes were cooled rapidly in water. They were inoculated with a washed suspension of *E. coli* grown in peptone

broth for 18 hours at 28° C., and incubated overnight (16 hours) while being shaken mechanically at 28° C. Turbidity measurements were made at a wavelength of 605μ using a Coleman spectrophotometer.

EXPERIMENTS AND RESULTS.

1. *The effect of copper on the response to vitamin B_{12}.*

The addition of copper sulphate to the basal medium caused a diminution in the growth response to vitamin B_{12} (Fig. 1a). In the absence of copper, maximum growth occurred when 1·0 mμg. B_{12} per tube was provided. When the concentration of copper in the final solution was 0·0002 p.c. the growth response to 1·0 mμg. B_{12} was reduced to that normally due to less than 0·5 mμg., while with 20 times this amount of copper the response to 1·0 mμg. B_{12}

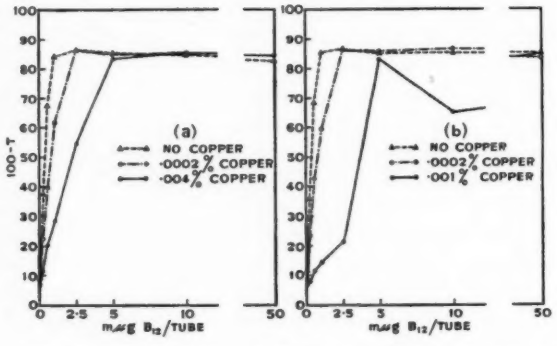

Fig. 1. The response of *E. coli* to vitamin B_{12} in the presence of (a) copper; (b) thioglycollic acid and copper.

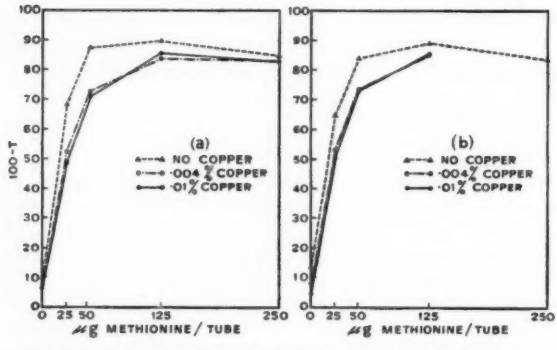

Fig. 2. The response of *E. coli* to methionine in the presence of (a) copper; (b) thioglycollic acid and copper.

was less than would be expected with 0·2 mμg. With increasing amounts of vitamin B_{12}, however, the inhibition due to copper was overcome and maximal growth was eventually obtained.

2. *The effect of copper on the response to methionine.*

In the absence of copper, the requirements of the test organism for methionine were approximately 50,000 times its requirements for vitamin B_{12}, and maximum growth occurred in the presence of 50 μg. of methionine per tube. The addition of copper had a much smaller effect on growth when the organism was using methionine in place of vitamin B_{12} (Fig. 2a). Copper at a concentration of 0·004 p.c. caused an inhibition slightly less than that due to one twentieth of this amount when vitamin B_{12} was the growth factor, while an increase in the concentration of copper up to 0·01 p.c. resulted in very little additional inhibitory effect. Increasing amounts of methionine overcame the inhibitory action of copper, and maximal growth was finally obtained at each copper level. At concentrations greater than 0·01 p.c. copper, precipitation occurred during autoclaving.

3. *The influence of cystine, homocystine and homocysteine.*

Homocystine and homocysteine were added to the basal medium in the same concentration as the other amino acids (0·02 p.c., or 0·01 p.c. in the final solution). The amount of cystine was adjusted to the same molecular concentration as homocystine and 0·0176 p.c. was used (0·0088 p.c. in the final solution).

Increasing concentrations of copper in the normal medium which contained no organic sulphur compounds caused a progressively greater inhibition of the response to vitamin B_{12}. All three sulphur compounds markedly protected the response of the organism against this inhibition (Fig. 3a, c and e). There were, however, some curious differences between their effects at the lower levels of copper. In the presence of cystine (Fig. 3a), the inhibition due to copper increased up to a concentration of 0·0001 p.c., but then suddenly diminished, so that the effect of 0·0002 p.c. copper was similar to that of one-tenth of this amount. Thereafter the inhibition slowly increased again, not returning to the maximum until the copper concentration was about 0·0008 p.c. With homocystine (Fig. 3c) there was at first a rapid and then a more gradual increase in inhibitory effect with increasing concentrations of copper. With homocysteine (Fig. 3e) a stimulatory effect of small amounts of copper, reaching its peak at 0·0001 p.c., was followed by a steadily increasing inhibition.

In the absence of copper the addition of these compounds only slightly modified the response of the organism to vitamin B_{12} (Fig. 4a).

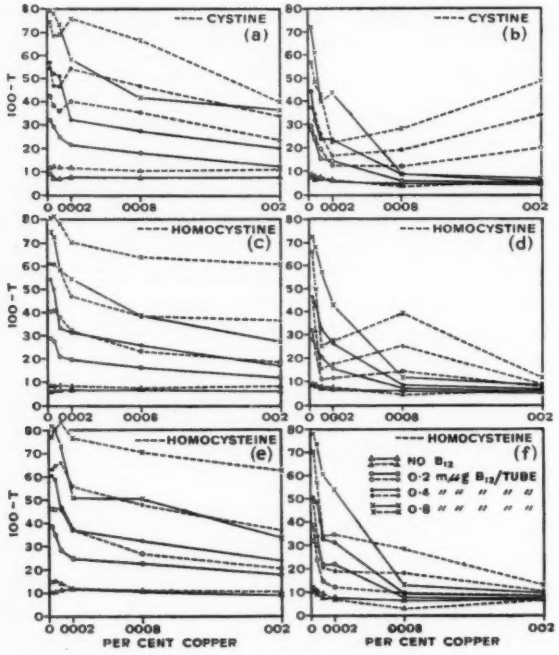

Fig. 3. The effect of increasing copper concentrations on the response of *E. coli* to vitamin B_{12}, as modified by the addition of cystine, homocystine and homocysteine to the medium.

(a) (c) and (e) no thioglycollic acid.
(b) (d) and (f) +thioglycollic acid.

Solid lines represent normal medium, dotted lines modified medium.

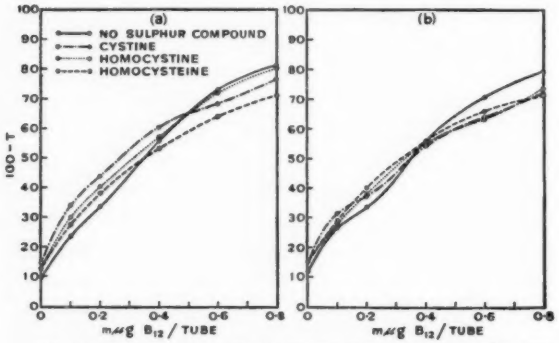

Fig. 4. The response of *E. coli* to vitamin B_{12} in the presence of (a) cystine, homocystine and homocysteine; (b) thioglycollic acid+cystine, homocystine and homocysteine.

4. *The influence of choline.*

The addition of choline (0·002 p.c.) to the basal medium was without any effect either on the normal response of *E. coli* to vitamin B_{12} or on the partial inhibition of this response by 0·0002 p.c. copper. This was the only concentration of copper tested for inhibitory effect in the presence of choline.

5. *The influence of thioglycollic acid.*

The medium proposed by Burkholder (1951), and normally used by us for the assay of vitamin B_{12} with this organism, contains thioglycollic acid (0·016 p.c.). Because of the known interaction of thioglycollic acid with copper (Bernheim and Bernheim, 1939), it was excluded from the medium used in the foregoing experiments. Similar tests were also performed, however, in the presence of thioglycollic acid.

i. *The effect of copper on the response to vitamin B_{12}.*

In the absence of copper and with a copper concentration of 0·0002 p.c., thioglycollic acid did not affect the growth response (Fig. 1b). In the presence of thioglycollic acid, however, the test organism exhibited much greater sensitivity to increasing copper concentration. Almost complete suppression of the growth response to 1·0 mμg. vitamin B_{12} was caused by 0·001 p.c. copper when the medium contained thioglycollic acid, while in its absence appreciable growth occurred with four times this concentration of copper (Fig. 1a).

Despite this enhanced effect of copper in the presence of thioglycollic acid, maximal growth of the organism ensued when sufficient vitamin B_{12} was supplied. No explanation can be offered for the dip in the response line with 0·001 p.c. copper after maximal growth had been attained.

ii. *The effect of copper on the response to methionine.*

In contrast to the results with vitamin B_{12}, the addition of thioglycollic acid did not affect the response to methionine at either level of copper tested (Fig. 2b). At higher dosage levels of methionine, however, heavy coagulation occurred in almost all the tubes containing copper and no readings could be obtained. The same phenomenon was observed in a few tubes among the lower levels of methionine, and also in some tubes in the corresponding test without thioglycollic acid.

iii. *The influence of cystine, homocystine and homocysteine.*

The addition of thioglycollic acid greatly modified the effects of these compounds on the inhibition of growth by copper (Fig. 3b, d and f). In the absence of thioglycollic acid the overall picture was one of a fair measure of protection against this inhibition (Fig. 3a, c and e). On the whole, the sulphur containing amino acids failed to afford a comparable degree of protection against the very much more severe effect of copper in the presence of thioglycollic acid.

The inhibition of growth response by the lower levels of copper was markedly aggravated by each of these compounds, although with higher concentrations of copper this was no longer the case. However, the effect of each compound at the higher copper levels was quite distinct. In the presence of cystine (Fig. 3b) the inhibitory effect of copper reached a maximum at 0·0002 p.c., thereafter diminishing so that at 0·002 p.c. copper the growth response resembled that in the presence of one-fortieth of this amount. With homocystine (Fig. 3d) the inhibition due to copper increased up to a concentration of 0·0001 p.c., but the growth response then steadily improved despite the increasing copper concentration until 0·0008 p.c. was reached. Past this point additional copper again resulted in increasing inhibition, and at 0·002 p.c. almost no protection due to homocystine was observed. When homocysteine was added (Fig. 3f) the growth response was progressively inhibited by increasing concentrations of copper, although at a somewhat different rate from that in the absence of homocysteine. There was at first a rapid and then more gradual increase in inhibitory effect. At the final level of 0·002 p.c. copper the presence of homocysteine had very little effect on the growth response.

In the absence of copper, the addition of thioglycollic acid to the medium did not alter the effect of these compounds on the response of the organism to vitamin B_{12} (Fig. 4b).

iv. *The influence of choline.*

Choline behaved in exactly the same way in the presence of thioglycollic acid as in its absence, i.e., it was without effect on either the normal response to vitamin B_{12} or on the inhibition caused by 0·0002 p.c. copper.

DISCUSSION.

The inhibitory effect of copper on *E. coli* was very much greater when growth was dependent on vitamin B_{12} than when methionine was the growth factor supplied: the reduction in growth with methionine caused by the addition of 0·004 p.c. copper was slightly less than that due to one-twentieth of this amount in the presence of vitamin B_{12}. Furthermore, while even larger amounts of copper resulted in little further depression of the growth response to methionine, increasing concentrations of copper very greatly reduced growth in the presence of vitamin B_{12}. It is difficult to avoid the conclusion that copper exerted its effect at some stage of the synthesis of methionine in which vitamin B_{12} is concerned.

In view of the ability of copper to catalyse the oxidation of sulphydryl groups (Pirie, 1931; Voegtlin, Johnson and Rosenthal, 1931; Rosenthal and Voegtlin, 1933; Kharasch, Legault, Wilder and Gerard, 1936; Bernheim and Bernheim, 1939), it might be supposed to inhibit the reduction of homocystine to homocysteine. Excess of vitamin B_{12}, which is believed to be concerned in

the maintenance of homocysteine in the reduced state (Dubnoff, 1952), might be expected to overcome the effect of copper and permit the reduction to take place: an excess of vitamin B_{12} did, in fact, overcome the inhibitory effect of copper on the growth of *E. coli* (Fig. 1).

If copper merely inhibited the reduction of homocystine, an adequate supply of homocysteine should permit a normal response to vitamin B_{12}. While in the absence of thioglycollic acid homocysteine did partially protect the organism's response to vitamin B_{12} from the inhibition due to copper, cystine and homocystine gave a similar degree of protection (Fig. 3). Tests with these compounds were carried out to indicate whether any effect which might be observed with homocysteine was due to its organic sulphur content, to its nature as a member of the homocystine-homocysteine cycle, or was due specifically to homocysteine itself. In these experiments no attempt was made to establish reducing conditions in the assay tubes. Some oxidation of homocysteine may therefore have occurred. However, certain differences between the growth responses in the presence of homocystine and homocysteine (Fig. 3) suggest that part at least of the homocysteine was available in the reduced form. In the presence of thioglycollic acid, itself a reducing agent, the protection afforded by homocysteine was greatly diminished at the higher concentrations of copper, while at the lower levels the inhibitory effect of copper was exacerbated.

It is possible that part of the action of copper is attributable to interference with the methylation of homocysteine. If copper is assumed to inhibit by interference with the action of vitamin B_{12}, this, in turn, would suggest that vitamin B_{12} must be implicated in the methylation step, either by direct participation in transmethylation or by catalysing the formation of methyl groups. This is in agreement with the findings of Shive (1950) and Gibson and Woods (1952) for *E. coli*. Working with rats, du Vigneaud, Ressler and Rachele (1950) and Arnstein and Neuberger (1953) have obtained evidence suggesting the involvement of vitamin B_{12} in methyl group synthesis. The fact that added choline failed to overcome or in any way mitigate the inhibition caused by 0·0002 p.c. copper provides some indication that any defect in the methylation system is unlikely to be due to a failure to produce choline as a source of methyl groups.

Dubnoff (1952) reported that under strict reducing conditions, homocysteine would support the growth of an *E. coli* mutant that requires either vitamin B_{12} or methionine. He has recently (1954) described several variants of the mutant which differed in their ability to use homocysteine under the conditions of his assay. Those variants with the highest requirements for vitamin B_{12} would grow only if supplemented with vitamin B_{12} or with methionine. The "high requirement," namely, 0·2 mμg. vitamin B_{12} per ml. for half maximum growth, reported by him, corresponds fairly closely to the needs of the organism used in these experiments. Under our assay conditions the amount of homocysteine supplied (500 μg. per tube) was equivalent to ten times the amount of

methionine necessary for maximum growth, and, if the organism could have used it, should have resulted in maximum growth at all levels of vitamin B_{12}. Since this did not occur, it would seem that homocysteine cannot replace methionine as a growth factor for this organism.

Thioglycollic acid was a constituent of our original medium as of Burkholder's (1951). Its presence in some of these experiments has introduced complications because of its known interaction with copper ions (Kharasch *et al.*, 1936; Bernheim and Bernheim, 1939), and because of its organic sulphur content. Its addition has in fact had a profound influence on many of the experiments reported here, the most striking being the greatly increased toxicity of higher concentrations of copper for the growth response to vitamin B_{12} (Fig. 1), and the diminished ability of the sulphur containing amino acids to exert a protective rôle against copper inhibition (Fig. 3).

Nevertheless, the main findings of this work, namely, that the inhibition by copper of growth dependent on vitamin B_{12} requires only a fraction of the copper concentration to depress growth response to methionine, and that this inhibition can be overcome by increasing amounts of the growth factor but not by choline or homocysteine, are the same whether or not thioglycollic acid is present.

No explanation can be offered for the extraordinary fluctuations in inhibitory effect of copper in the presence of the sulphur compounds. It is apparent that the reactions involved must be very complex, and that this assay procedure is not a suitable instrument for their further investigation.

SUMMARY.

The addition of copper to the medium inhibited the growth of a mutant of *E. coli* requiring vitamin B_{12} or methionine. The reduction in growth with methionine caused by 0·004 p.c. copper was slightly less than that due to one-twentieth of this amount in the presence of vitamin B_{12}. Increasing amounts of copper reduced growth on vitamin B_{12} very greatly, but had little effect on the growth response to methionine. Excess vitamin B_{12} or methionine overcame the inhibition and permitted normal growth.

The provision of a large excess of homocysteine (10 times the amount of methionine for maximal growth) did not restore a normal response to vitamin B_{12} in the presence of copper, although some protection of the growth response was afforded by homocysteine, homocystine or cystine. The addition of choline had no effect on the inhibition due to one concentration of copper (0·0002 p.c.).

The presence of thioglycollic acid in the medium resulted in a greatly increased toxicity of higher concentrations of copper for the growth response to vitamin B_{12} but not methionine. There was also a marked diminution in protection by the sulphur compounds against copper toxicity.

It is suggested that copper exerted its effect at some stage or stages of the synthesis of methionine in which vitamin B_{12} is concerned.

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THE TOXICITY OF HEAVY METALS TO MAMMALIAN SPERMATOZOA

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Few systematic observations have been made on the effect of heavy metals on spermatozoa although they might be expected to be toxic since many enzymes, particularly those containing essential sulphydryl groups, are inhibited by these elements (see Sumner and Myrback, 1950).

Most manuals of artificial insemination practice (e.g., Anderson, 1946; Goode and Ruddock, 1948) prescribe the use of distilled water in making up diluents, presumably to avoid metallic contamination. It may be noted in this connection that Dubincik (1934) states that iron and zinc are injurious to spermatozoa. Mercury (Baker, 1931) and copper (Macleod, 1947 and 1951) have also been reported harmful to human spermatozoa, and Emmens and Swyer (1948) found the latter metal depressed the motility of rabbit spermatozoa after several hours.

There is also evidence that trace concentrations of heavy metals are toxic to diluted sea urchin spermatozoa (Tyler and Rothschild, 1951; Tyler, 1953) and that they can inhibit or produce abnormal fertilization in invertebrate eggs (Lillie, 1921; Hoadley, 1923; Runnstrom, 1939; Cleland, 1953).

The purpose of this paper is to present a comparative study of the effect of some heavy metals (Mn^{++}, Fe^{++}, Co^{++}, Cd^{++}, Zn^{++}, Cu^{++}, Pb^{++}) on ram, bull, rabbit and human spermatozoa.

Materials and Methods.

The method of collecting semen and the composition of the diluting fluid were the same as described previously (White, 1954a).

Semen was diluted 1 to 40 in small tubes for the motility studies which were made at room temperature.

Motility was scored at hourly intervals over a 4-hour period by the system of Emmens (1947). Full motility was rated as 4 and complete immotility as zero, but in presenting results the actual scores have been multiplied by 4, since quarter-grades were frequently used. All tests were repeated on 4 ejaculates.

The metals were added as the AR sulphate salts with the exception of lead, which was added as the acetate.

Glutathione was supplied by Schwarz Laboratories, cysteine by British Drug Houses, and British Anti-Lewisite (BAL) by Boots Pure Drug Co.

Statistical analysis.

The results have been subjected to standard analysis of variance (see Fisher, 1946) with isolation of sums of squares attributed to differences between ejaculates and treatments. The total motility score (×4) has been used as unit observation and the interaction mean square as the error term. Differences between ejaculates are often highly significant, so that the accuracy of comparisons is much improved by analysis in this way. The standard error of the difference between a pair of treatment means has been calculated using the formula

$$sd = \frac{2s^2}{r}$$

where sd=standard error of the difference between a pair of treatment means

s^2=interaction mean square

r=number of replicates.

The significance of differences between pairs of treatment means has then been assessed by the t-test using sd and the degrees of freedom associated with s^2.

Summaries of the analyses of variance are presented below the data in the Tables, and show variance ratios with the interaction means square in italics at the base of the columns.

RESULTS.

The effect of three concentrations (2·00, 0·20 and 0·02 mM) of manganese, iron, cobalt, copper, zinc, cadmium and lead on the motility of ram, bull, rabbit

TABLE 1.

The effect of some heavy metals on the total motility score (×4) of ram, bull, rabbit and human spermatozoa over a 4 hr. period. Each value represents the mean of four ejaculates.

Metal	Concentration mM	Ram	Bull	Rabbit	Human
Nil	—	58	54	62	47
Manganese	2·00	3	51	57	45
	0·20	57	54	63	49
	0·02	58	55	63	47
Iron	2·00	16**	41**	30**	25**
	0·20	50**	53	57	45
	0·02	58	55	63	46
Cobalt	2·00	54	52	44**	39*
	0·20	57	54	63	47
	0·02	57	54	62	46
Copper	2·00	21**	50*	28**	32**
	0·20	28**	51	35**	42
	0·02	45**	52	51	43
Zinc	2·00	53	52	64	36**
	0·20	55	56	63	46
	0·02	58	55	60	45
Cadmium	2·00	57	48**	59	15**
	0·20	58	51	63	25**
	0·02	58	53	62	39*
Lead	2·00	53	51	53*	23**
	0·20	58	54	58	45
	0·02	58	55	63	47

* Significantly toxic $P<0·05$.
** Highly significantly toxic $P<0·01$.

Summary of Analysis of Variance.

Source of variation	D.F.	Variance ratios			
		Ram	Bull	Rabbit	Human
Between treatments	21	40·3**	6·3**	15·7**	14·1**
Between ejaculates	3	55·9**	50·3**	10·1**	34·6**
Interaction	63	*15*	*6*	*30*	*26*

** $P<0·01$.

and human spermatozoa are set out in Table 1. Analyses of variance show highly significant variation between treatments for each species.

The standard error of the difference between two treatments means is in each case the square root of half the interaction mean square. With 63 degrees of freedom t must be greater than 2·0 for significance at the 5 p.c. level and greater than 2·7 at the 1 p.c. level. From this it can be calculated that the treatment means must differ from the controls by the following approximate minimum values if an effect is to be judged significant.

	Probability level	
	0·05	0·01
Ram	6	8
Bull	4	5
Rabbit	8	11
Human	8	10

Manganese. Manganese was harmless to all spermatozoa at the concentrations tried.

Iron. Iron was highly significantly toxic to ram spermatozoa at 2·00 and 0·20 mM and to bull, rabbit and human spermatozoa at the highest level. An effect was usually seen within one or two hours, by which time ram spermatozoa were completely immobilized by the highest concentration. The effect of iron on rabbit and human spermatozoa was, however, less pronounced and with bull spermatozoa even slighter.

Cobalt. The motility of rabbit and human spermatozoa was depressed by 2·00 mM cobalt. No significant effect was seen on the other spermatozoa, and even with the above two species the effect of cobalt was often not very marked.

Copper. Copper was toxic to ram spermatozoa even at the lowest concentration and in many cases caused head agglutination. Viability decreased after one to three hours in copper-containing diluents and the spermatozoa were usually immotile after four hours.

At 2·00 and 0·20 mM, copper also depressed the motility of rabbit spermatozoa after about an hour, but some cells were usually still progressing at the end of the experiment.

The scores for bull and human spermatozoa in 2·00 mM copper were also significantly less than the controls, but the effect was not marked with the first species and was variable with the second.

Zinc. The highest concentration of zinc (2·00 mM) was significantly harmful to human spermatozoa, but again ejaculates differed considerably in their susceptibility. No other effects were seen.

Cadmium. Cadmium was toxic to human spermatozoa at all three concentrations. In the presence of 2·00 mM cadmium, motility was markedly depressed after one hour and all cells were immotile after 2 hours. At 0·20 mM and 0·02 mM an effect was seen after two hours and usually the higher of these concentrations completely immobilized human spermatozoa within four hours.

The motility of bull spermatozoa was also significantly depressed by 2·00 mM but the effect was not striking.

Lead. Lead at the highest concentration caused a slight drop in the motility of rabbit and human spermatozoa usually after an hour. It had no significant effect on the spermatozoa of the other species.

Effect of sulphydryl compounds on heavy metals.

The inhibition of many enzymes by heavy metals is believed to be due to their combination with essential sulphydryl groups. Such enzymes can often

TABLE 2.

The effect of cysteine, glutathione and BAL on the total motility score (×4) of ram and human spermatozoa. Each value represents the mean of four ejaculates.

Species	Control	Cysteine conc. (mM)			Glutathione conc. (mM)			BAL conc. (mM)		
		1·6	0·8	0·4	1·6	0·8	0·4	1·6	0·8	0·4
Ram	61	65	63	64	65	64	64	53*	56	60
Human	49	32**	30**	35**	36**	43	49	28**	28**	29**

* Significantly toxic $P<0·05$.
** Highly significantly toxic $P<0·01$.

Summary of Analysis.

Source of variation	D.F.	Variance ratio	
		Ram	Human
Between treatments	9	30·2**	8·5**
Between ejaculates	3	54·7**	15·7**
Interaction	*27*	*21*	*42*

** $P<0·01$.

be protected by the additions of thiols. In general, BAL is the most powerful agent, glutathione comes next, and finally cysteine (Barron, 1951). It was of interest, therefore, to see if these compounds modified the toxicity of the heavy metals for spermatozoa.

The systems chosen for the tests were copper and cadmium with ram and human spermatozoa respectively. Preliminary experiments were necessary to establish the levels of cysteine, glutathione and BAL that could be used with impunity since it has been found previously (White, 1954b) that high concentrations can be toxic to diluted spermatozoa. The results are set out in Table 2. For a significant depression of motility, treatments means must differ from the controls by the following minimum values.

	Probability level	
	0·05	0·01
Ram	7	10
Human	10	13

BAL at 1·6 mM was the only treatment that proved harmful to ram spermatozoa. However, human spermatozoa were severely affected by all concentrations of cysteine and BAL and by the highest level of glutathione.

TABLE 3.

The effect of 1·6 mM cysteine, 1·6 mM glutathione and 0·8 mM BAL, singly and in combination, on the toxicity of 0·2 mM copper for ram spermatozoa. Values represent the mean total motility score (×4) over a 4 hr. period for four ejaculates.

Control	Nil	Glutathione	Cysteine	BAL	Glutathione and cysteine	Glutathione and BAL	Cysteine and BAL	Glutathione and cysteine and BAL
54	18	48	11	13	30	57	29	52

Summary of Analyses of Variance.

Source of variation	D.F.	Variance ratio
Between treatments:	7	31·4**
Effect of glutathione	1	162·0**
Effect of cysteine	1	1·8
Effect of BAL	1	19·9**
G/C interaction	1	10·5**
G/B interaction	1	3·5
C/B interaction	1	12·6**
G/C/B interaction	1	1·5
Between ejaculates	3	9·3**
Interaction (residual)	*21*	*41*

** $P<0·01$.

Table 3 shows the effect of 1·6 mM glutathione, 1·6 mM cysteine and 0·8 mM BAL, singly and in combination, on the toxicity of 0·2 mM copper for ram spermatozoa. It is clear from the summary of the factorial analysis that glutathione is highly effective in countering the toxic action of copper. Although alone, cysteine and BAL had no effect on motility, in combination they slightly improved it, as can be seen from the high cysteine/BAL interaction. This accounts for the significant main effect of BAL in the analysis of variance. Cysteine and glutathione also interacted but in the opposite direction, the motility score in the presence of the two being somewhat less than might be expected from their separate effects. Both compounds prevented the marked head agglutination which occurs when ram spermatozoa are diluted in copper-containing solutions.

Cysteine and BAL were obviously too toxic to be of much use with human spermatozoa; 0·8 mM glutathione, however, was found to be effective in decreasing the spermicidal action of 0·2 mM cadmium. This can be seen from the results of the factorial experiment and the summary analysis of variance set out in Table 4.

TABLE 4.

The effect of 0·2 mM cadmium and 0·8 mM glutathione on the total motility score (×4) of human spermatozoa over a 4 hr. period. Each value represents the mean of four ejaculates.

Control	0·2mM Cadmium	0·8mM Glutathione	0·2mM Cadmium and 0·8mM Glutathione
43	21	42	37

Summary of Analysis of Variance.

Source of variation	D.F.	Variance ratio
Between treatments	3	27·1**
Effect of cadmium	1	47·3**
Effect of glutathione	1	16·0**
C/G interaction	1	18·1**
Between ejaculates	3	34·4**
Interaction (residual)	*9*	*16*

** $P<0·01$.

DISCUSSION.

Iron and copper were the most consistently spermicidal of the heavy metals tried, but the levels required to cause toxicity were in some cases relatively high and the effects, even at these concentrations, were not always marked. Copper in these experiments was more toxic to rabbit spermatozoa than was found by Emmens and Swyer (1948) and less toxic to human spermatozoa than was found by Macleod (1951).

The following levels have been recommended as safe maxima for the heavy metal content of water for human consumption (Anon, 1936):

Lead	0·1 p.p.m. (0·0005 mM)
Copper	0·2 p.p.m. (0·0031 mM)
Zinc	5·0 p.p.m. (0·0769 mM)
Iron	0·3 p.p.m. (0·0054 mM)

The heavy metal content of most water supplies is usually much less (Suckling, 1943) and in any case with the exception of zinc, which is not spermicidal except in very high concentrations, these maximum levels are well below the minimum (0·02 mM) used in these experiments. It is unlikely that the heavy metals in such water would ever depress the motility of spermatozoa and it would seem unnecessary to use distilled water in diluents for artificial insemination in order to avoid metallic contamination.

The two most striking effects were the toxicity of copper for ram spermatozoa and that of cadmium for human spermatozoa. The protective action of glutathione in each case is interesting in view of the work of Macleod, 1951, and the discovery that glutathione is the coenzyme of glyceraldehyde phosphate dehydrogenase (Racker and Krimsky, 1952; Krimsky and Racker, 1952). It is tempting to suggest that the spermicidal effect of copper and cadmium is due to the inactivation of this enzyme system.

SUMMARY.

In studies over 4 hours at room temperature on the effect of heavy metals (Mn^{++}, Fe^{++}, Co^{++}, Cd^{++}, Cu^{++} Zn^{++} and Pb^{++} at 2·00, 0·20 and 0·02 mM) on the motility of ram, bull, rabbit and human spermatozoa, it was found that:

Iron and copper were the most consistently toxic, although there were large species differences; thus bull spermatozoa were little affected by copper whilst ram spermatozoa were particularly susceptible.

Cadmium was toxic to human spermatozoa and slightly so to bull spermatozoa.

Lead and cobalt in high concentrations tended to depress the motility of rabbit and human spermatozoa.

Glutathione decreased the toxicity of copper for ram spermatozoa and cadmium for human spermatozoa.

It is suggested that it is unnecessary to use distilled water in diluents for artificial insemination in order to avoid metallic contamination.

Acknowledgments. I am indebted to Professor C. W. Emmens for his interest and advice; to Mr. A. W. Blackshaw for collecting ram serum; to the University Farm and Camden Park Estate for bull semen; and to the Women's Hospital, Sydney, for the supply of human semen.

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THE COLLECTION OF RABBIT SEMEN

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The rabbit is a convenient laboratory animal for the study of reproductive physiology and the male provides a ready source of material for the investigation of mammalian semen.

The purpose of this communication is to describe an artificial vagina and attachments that have been used successfully for some time in this laboratory for the collection of rabbit semen.

Construction of the artificial vagina.

The artificial vagina is an improved version of that described by Macirone and Walton (1938). It consists of the following (see Fig. 1):

$3\frac{1}{2}$″ length of rubber hose (A) of internal diameter $1\frac{1}{2}$″ and $\frac{1}{8}$″ wall thickness.

$3\frac{1}{2}$″ diameter metal stopper (B) with long (C) and short (D) pieces of $\frac{1}{4}$″ diameter copper tubing and with a $1\frac{1}{2}$″ screw thread hole through it.

$1\frac{1}{2}$″ long metal tube (E) with an outer screw thread at one end to fit the hole in the stopper (B) and with a slightly increased internal diameter commencing $\frac{3}{4}$″ from this end.

6″ long tapering rubber sheath (F) of $1\frac{1}{2}$″ greater diameter and $\frac{3}{4}$″ lesser diameter. These were supplied by the Dunlop Rubber Coy., and were boiled for several minutes before initial use in order to remove spermicidal substances.

$\frac{3}{4}$″ diameter test-tube cut off $1\frac{1}{2}$″ from its base (G).

The artificial vagina is assembled in the following way:

1. The metal stopper is fitted to the rubber hose so that the long copper tube lies within the hose.
2. A sheath is placed in the hose and the smaller end pulled through the hole in the stopper.
3. The metal tube is screwed into the hole in the stopper so that it projects on the opposite side to the hose. A washer may be necessary to seal the joint.
4. The ends of the sheath are turned back over the hose and metal tube, and secured by rubber bands.
5. Short lengths of rubber tubing are fitted to the outside ends of tubes C and D and closed off with a spring clip (H).

The metal tube protects the collection tube from breakage and its narrowing internal diameter stops the collection tube from moving up and also enables the inside of the lip of the latter to lie flush with the rubber sheath. This, together with the use of the wide bore collection tube, allows a much freer flow of semen than in the model of Macirone and Walton (1938). Another advantage of the present vagina is that, when used in conjunction with the apparatus described in the next section, it is only necessary to manipulate one spring clip in order to fill it with water.

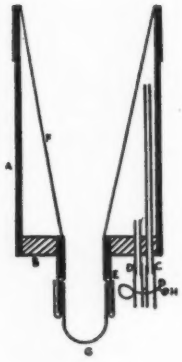

Fig. 1.
Artificial vagina for the collection of rabbit semen.

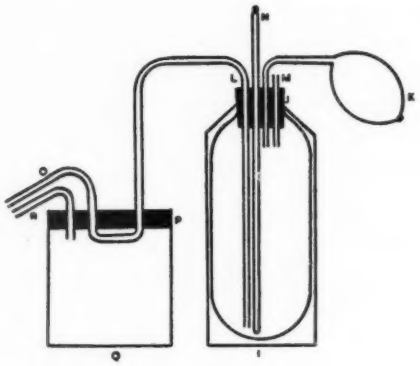

Fig. 2.
Apparatus for filling the artificial vagina.

Apparatus for filling artificial vagina with water.

The following apparatus (Fig. 2) has been evolved which enables the artificial vagina to be filled quickly and easily.

It consists of a thermos flask (I) fitted with a stopper (J), a rubber bulb (K), an outlet tube (L), an escape tube (M) and a thermometer (N). The outlet tube is connected by rubber tubing to the curved tube (O) which is held in position by a loose fitting cork (P) in the jar (Q). A second curved tube (R) is fitted to the cork.

Water at about 50° C. is stored in the thermos flask and forced into the space between the rubber sheath and the hose of the artificial vagina. This is done by releasing the spring clip, connecting tubes C and D to O and R respectively, and squeezing the rubber bulb with one finger over the escape tube. When water runs from the tube (R) pumping is stopped, the finger taken off the escape tube and tubes C and D sealed off with the spring clip.

Method of collecting semen.

The artificial vagina (Fig. 1) is disconnected from the filling apparatus (Fig. 2) and petroleum jelly applied lightly to the inside of the sheath with a glass rod.

The collection tube (G) is placed inside the metal tube and the artificial vagina held between the hind legs of the doe.

When the male mounts, his penis enter the open, lubricated end of the vagina and ejaculation occurs. In order to obtain rapid ejaculation the sheath must be kept warm and it is usually necessary to pump hot water from the thermos through the vagina after every second or third collection.

Once a buck is trained to use the artificial vagina, the teaser doe may be dispensed with and the collections made with a dummy. This is a rabbit skin glove, worn fur outwards on the hand that holds the artificial vagina. An even more convenient practice when collecting a number of specimens is to take two males from their cages at one time and allow them to mount each other.

After use, the artificial vagina should be dismantled and the rubber sheath rinsed with water and dusted with French chalk. All the French chalk must be washed off and the sheath dried, preferably over a compressed air jet, before another collection.

SUMMARY.

An improved artificial vagina and filling apparatus is described for the collection of rabbit semen.

Acknowledgment. I am indebted to Mr. J. Tye for his valuable technical assistance.

REFERENCE.

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LYSOGENICITY AND LYSIS PATTERNS IN THE SALMONELLAS

3. A BACTERIOPHAGE GROUPING SCHEME FOR *S. ADELAIDE*

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(Accepted for publication 8th March, 1955.)

Atkinson and Geytenbeek (1953) suggested four groups for strains of *S. adelaide* depending on phage carriage and lysis pattern with three type phages. The lysis pattern was related to phage carriage. Later Atkinson and Klauss (1954) described enlarged lysogenicity and lysis patterns for 89 strains of *S. adelaide*, using the three type phages and two additional new phages, as well as two indicator strains to detect lysogenicity. From this work a phage grouping scheme for *S. adelaide* has been developed and is presented in detail here.

METHODS.

The lysis tests were carried out according to the method originally described by Craigie and Yen (1938) for Vi phage typing of *S. typhi* except that the phage preparations were used undiluted on account of their low titres. Serial propagation of phages was avoided as it tended to produce mutants with different lytic patterns from the parent strains.

The lysogenicity tests were done by growing the test strain and the indicator strain together on an agar plate and extracting the mixed growth with broth. This extract was centrifuged until clear and then used in a lysis test on the indicator strain from which it was prepared. If the extract produced lysis then the test strain was lysogenic on that indicator strain and the result was recorded as + (see Table 1). If the extract produced no lysis then the test strain was considered non-lysogenic on that indicator strain and the result was recorded as − (see Table 1).

The medium used in these tests was Oxoid nutrient broth with 10 p.c. Oxoid yeast extract, solidified with 1 p.c. agar.

THE PHAGE GROUPING SCHEME.

The scheme contained four main groups, 1, 2, 3 and 4, of which groups 1 and 2 were each divided into two sub-groups, 1a and 1b, 2a and 2c. The groups were defined by both lysis and lysogenicity. Strains to be grouped were tested for lysis with six type phages and for lysogenicity with two indicator strains.

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The six type phages were Ad2 (type 1a), Ad20 (type 1b), Ad37H (type 2a), Ad37C (type 2b), Ad273 (type 2c), Ad14 (type 3). The first four phages represented the four types (1a, 1b, 2a, 2b) of *S. adelaide* phage described by Atkinson and Geytenbeek (1953). The other two phages, Ad273 and Ad14, were briefly described, together with others like them, by Atkinson and Klauss (1954). They represented two new types of *S. adelaide* phage provisionally labelled types 2c and 3. These two new types will be described in a separate paper. The two indicator strains used in the scheme were 14 and Cey4. Strain 14, described by Atkinson and Geytenbeek (1953), was sensitive to all the type phages except Ad14 (type 3) and thus detected lysogenic strains carrying any of these phages. Strain Cey4, described by Atkinson and Klauss (1954), was sensitive to all the type phages and was included to detect lysogenic strains carrying only type 3 phage which could not be detected by strain 14.

The typical lysis and lysogenicity patterns for each of the six groups are given in Table 1, which presents the results of applying the scheme to the type strain of each group. The type strains of groups 1a, 1b, 2a and 3 were described by Atkinson and Geytenbeek (1953) and were strains 2, 20, 37 and 14 respectively. The type strains of groups 2c and 4 were described by Atkinson and Klauss (1954) and were strains S273 and Cey4. Table 1 also gives the type of phage carried by each group (from the data of Atkinson and Geytenbeek, 1953, and Atkinson and Klauss, 1954).

TABLE 1

Reactions of type strains of *S. adelaide* in the proposed phage grouping scheme.

Proposed group no.	Type strain no.	Phage used in lysis test						Lysogenicity with strain		*S. adelaide* phage type carried
		Ad2 (type 1a)	Ad20 (type 1b)	Ad37H (type 2a)	Ad37C (type 2b)	Ad273 (type 2c)	Ad14 (type 3)	14	Cey4	
1a	2	−	+	+	+	+	−	+	+	1a and 3
1b	20	+	−	+	+	+	−	+	+	1b and 3
2a	37	+	+	−	−	+	−	+	+	2a and 3
2c	S273	+	+	−	−	−	−	+	+	2c and 3
3	14	+	+	+	+	+	−	−	+	3
4	Cey4	+	+	+	+	+	+	−	−	none

+ = confluent or semi-confluent lysis or plaques.

In this phage grouping scheme, group 1 was characterized by (a) its sensitivity to type 2 phages (Ad37H, Ad37C and Ad273), and its resistance to type 3 phage (Ad14), (b) its lysogenicity with both indicator strains 14 and Cey4. Group 1 was sub-divided into groups 1a and 1b on sensitivity to type 1 phages. Group 1a was sensitive to type 1b phage (Ad20) and resistant to type 1a phage (Ad2). Group 1b was sensitive to type 1a phage (Ad2) and resistant to type 1b phage (Ad20).

Group 2 was characterized by (a), its sensitivity to type 1 phages (Ad2 and Ad20) and its resistance to type 3 phage (Ad14), (b) its lysogenicity with both indicator strains 14 and Cey4. Group 2 was subdivided into groups 2a and 2c on sensitivity to type 2 phages. Group 2a was sensitive to type 2c phage (Ad273) and resistant to types 2a and 2b phages (Ad37H and Ad37C). Group 2c was resistant to all the type 2 phages (Ad37H, Ad37C, Ad273).

Group 3 was characterized by (a) its sensitivity to type 1 and type 2 phages and its resistance to type 3 phage (Ad14), (b) its lysogenicity with the indicator strain Cey4 and its failure to show lysogenicity with the indicator strain 14.

Group 4 was characterized by (a) its sensitivity to all the six type phages, (b) its failure to show lysogenicity with either indicator strain 14 or Cey4.

Group 1 and group 2 both carried two phages consisting of type 3 phage and a type 1 or type 2 phage characteristic of the group. Thus, in group 1a, the characteristic phage was type 1a, in group 1b it was type 1b, in group 2a it was type 2a, and in group 2c it was type 2c. Group 3 carried only type 3 phage and group 4 carried none of the type phages. The lysis and lysogenicity patterns, as shown in Table 1, were sufficient to identify the group.

DISCUSSION.

The proposed scheme was purposely called a phage grouping scheme with subdivisions called groups, as distinct from a phage typing scheme with subdivisions called types. This distinction was made because the groups of the *S. adelaide* phage grouping scheme depended for their definition not only upon lysis pattern with type phages, but also upon lysogenicity. In the phage typing schemes for *Staph. aureus* (Williams and Rippon, 1952), *S. typhi* (Craigie and Felix, 1947) and *S. paratyphi B* (Felix and Callow, 1943), the types were distinguished by their lysis patterns with the type phages.

In the *S. adelaide* phage groups the lysis patterns were closely related to the type of phage carried. Thus in groups 1a, 1b, 2a and 3, the lysis pattern was a direct reflection of the carried phages to which these groups were resistant. In group 2c, however, resistance occurred not only to the carried phages but also to the related type 2a and type 2b phages. Possibly stronger preparations of the type 2a and type 2b phages than at present available might lyse the group 2c strains. In group 1a, lysis by type 1b phage depended upon the phage concentration, and weak preparations might fail to lyse group 1a strains. Apparently carriage of type 1a phage rendered the strain less sensitive to type 1b phage than other strains not carrying any type 1 phage. However, carriage of type 1b phage by group 1b strains failed to interfere with their lysis by type 1a phage to which they were just as sensitive as strains not carrying any type 1 phage.

By introduction of the lysogenicity test into the grouping scheme, strains giving the lysis pattern of a group but failing to show the group lysogenicity pattern were excluded from any group. One such strain, no. 67, was described by Atkinson and Klauss (1954), who obtained the lysis pattern of group 3 but could not detect lysogenicity.

The phage grouping scheme described here has been applied to 413 strains of *S. adelaide* by Atkinson and Klauss (1955) and only 21 of these strains failed to fit into any of the six groups.

SUMMARY.

A bacteriophage grouping scheme for strains of *S. adelaide* was devised. The scheme contained six groups distinguished by (a) lysis pattern with six *S. adelaide* type phages, (b) lysogenicity tests with two indicator strains. The group patterns were closely related to phage carriage.

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LYSOGENICITY AND LYSIS PATTERNS IN THE SALMONELLAS

4. APPLICATION OF A BACTERIOPHAGE GROUPING SCHEME TO 413 STRAINS OF *S. ADELAIDE*

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A bacteriophage grouping scheme proposed by Atkinson (1955) and depending upon lysis and lysogenicity tests has been applied to 413 strains of *S. adelaide* comprising the complete collection of strains of this type held in this laboratory. More than half these strains came from Ceylon and the rest were mainly from various parts of Australia. All except a small number of strains fitted into the phage grouping scheme.

MATERIALS AND METHODS.

The 413 strains of *S. adelaide* consisted of 108 Australian strains numbered 1 to 28, 30 to 43, 45 to 67, 92 to 95, 104 to 108, 287 to 292 and 390 to 417; six American strains numbered 273 to 278; one English strain numbered 279; six Belgian Congo strains numbered 206 to 211; 292 Ceylon strains numbered 68 to 91, 96 to 103, 109 to 205, 212 to 272, 280 to 286 and 293 to 387.

The lysis and lysogenicity tests were carried out by the methods described for the phage grouping scheme by Atkinson (1955) using the six *S. adelaide* type phages, Ad2 (type 1a), Ad20 (type 1b), Ad37H (type 2a), Ad37C (type 2b), Ad273 (type 2c) and Ad14 (type 3), and two indicator strains 14 and Cey4.

EXPERIMENTAL.

The results of the lysis and lysogenicity tests on the 413 strains of *S. adelaide* are given in Table 1, which also includes the group to which each strain was allotted on these reactions.

The majority of the strains in Table 1 gave reactions characteristic of a group defined in the phage grouping scheme. The actual number of the strains falling into each of the six groups is given in Table 2 in which the strains are divided up according to source.

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TABLE 1.

Results with 413 strains of *S. adelaide* tested for lysis and lysogenicity according to the phage grouping scheme.

S. adelaide test strain nos.	Lysis test with phage type						Lysogenicity test on strain		Grouping of test strain
	1a (Ad2)	1b (Ad20)	2a (Ad37H)	2b (Ad37C)	2c (Ad273)	3 (Ad14)	14	Cey4	
Ceylon strains.									
68 to 91	+	+	+	+	+	+	−	−	group 4
96 to 103	+	+	+	+	+	+	−	−	group 4
109 to 203	+	+	+	+	+	+	−	−	group 4
204, 205	+	+	+	+	+	−	−	+	group 3
212 to 215	+	+	+	+	+	+	−	−	group 4
216	+	+	−	−	−	+	−	−	no group
217 to 220	+	+	+	+	+	+	−	−	group 4
221	−	−	−	pls	−	−	−	−	no group
222 to 227	+	+	+	+	+	+	−	−	group 4
228	+	+	−	−	−	+	−	−	no group
229 to 272	+	+	+	+	+	+	−	−	group 4
280 to 286	+	+	+	+	+	+	−	−	group 4
293 to 306	+	+	+	+	+	+	−	−	group 4
307	−	−	−	+	−	−	−	−	no group
308 to 344	+	+	+	+	+	+	−	−	group 4
345	−	−	−	+	−	−	−	−	no group
346, 347	+	+	+	+	+	−	−	−	no group
348	−	−	−	pls	−	−	−	−	no group
349	pls	pls	−	pls	−	−	−	−	no group
350	+	pls	−	+	pls	−	−	−	no group
351 to 356	+	+	+	+	+	+	−	−	group 4
357	pls	−	pls	+	pls	−	−	−	no group
358 to 387	+	+	+	+	+	+	−	−	group 4
Australian strains.									
1	+	+	+	+	+	−	−	+	group 3
2	−	+	+	+	+	−	+	+	group 1a
3 to 12	+	+	+	+	+	−	−	+	group 3
13	+	+	−	−	+	−	+	+	group 2a
14	+	+	+	+	+	−	−	+	group 3
15, 16	+	+	−	−	+	−	+	+	group 2a
17, 18, 19	+	+	+	+	+	−	−	+	group 3
20	+	−	+	+	+	−	+	+	group 1b
21 to 28	+	+	+	+	+	−	−	+	group 3
30, 31	+	+	+	+	+	−	−	+	group 3
32	−	pls	−	−	pls	−	−	−	no group
33 to 36	+	+	+	+	+	−	−	+	group 3
37	+	+	−	−	+	−	+	+	group 2a
38	+	+	+	+	+	−	−	+	group 3
39, 40	+	+	−	−	+	−	+	+	group 2a
41, 42	+	+	+	+	+	−	−	+	group 3
43, 45	+	+	−	−	+	−	+	+	group 2a
46	+	+	+	+	+	−	−	+	group 3
47	+	+	−	−	+	−	+	+	group 2a
48	+	+	+	+	+	−	−	+	group 3

+ = confluent or semi-confluent lysis.
pls = plaques.
− = no lysis.

TABLE 1—*continued.*

Results with 413 strains of *S. adelaide* tested for lysis and lysogenicity according to the phage grouping scheme.

S. adelaide test strain nos.	Lysis test with phage type						Lysogenicity test on strain		Grouping of test strain
	1a (Ad2)	1b (Ad20)	2a (Ad37H)	2b (Ad37C)	2c (Ad273)	3 (Ad14)	14	Cey4	
49	+	+	–	–	+	–	+	+	group 2a
50 to 66	+	+	+	+	+	–	–	+	group 3
67	+	+	+	+	+	–	–	–	no group
92 to 95	+	+	+	+	+	–	–	+	group 3
104, 105	+	+	+	+	+	–	–	+	group 3
106	+	+	–	–	+	–	+	+	group 2a
107, 108	+	+	+	+	+	–	–	+	group 3
287 to 292	+	+	+	+	+	–	–	+	group 3
390 to 392	+	+	+	+	+	–	–	+	group 3
393	phage contaminated								no group
394	+	+	–	–	+	–	+	+	group 2a
395 to 413	+	+	+	+	+	–	–	+	group 3
414	+	pls	–	–	pls	–	+	+	group 2a
415	–	pls	pls	+	pls	–	+	+	group 1a
416, 417	+	pls	pls	+	pls	–	–	+	group 3
American strains.									
*273 to 277	+	+	–	–	–	–	+	+	group 2c
*278	–	–	–	–	–	–	–	–	no group
English strain.									
*279	+	+	+	+	+	–	–	+	group 3
Belgian Congo strains.									
206 to 211	+	+	+	+	–	–	–	+	no group

+ = confluent or semi-confluent lysis.
pls = plaques.
– = no lysis.

* In earlier papers these numbers were S273 to S279.

The totals given in Table 2 show the numbers found in each group among the 413 strains. Only 21 strains failed to fit into a group. Group 4 contained the largest number of strains (279), all of which came from Ceylon. Group 3 contained the next largest number of strains (92), the majority (89) of which came from Australia. Group 2c contained five strains, all from America. The rarest groups were 1a and 1b, represented by two strains and one strain respectively, all from Australia.

The figures in Table 2 also show the distribution of strains from a particular locality in the phage groups. Of the Ceylon strains, 279 out of 292 gave the reactions of group 4, two gave the reaction of group 3, and eleven failed to give characteristic group reactions and were therefore ungrouped. Of the Australian strains, 89 fitted into group 3, 13 into group 2a, two into group 1a, one into

TABLE 2.

Allocation of 413 strains of *S. adelaide* to the six groups of the phage grouping scheme.

Number of strains tested	Number of strains allotted to						
	group 1a	group 1b	group 2a	group 2c	group 3	group 4	no group
Ceylon strains. 292	0	0	0	0	2	279	11
Australian strains. 108	2	1	13	0	89	0	3
English strain. 1	0	0	0	0	1	0	0
American strains. 6	0	0	0	5	0	0	1
Belgian Congo strains. 6	0	0	0	0	0	0	6
Totals 413	2	1	13	5	92	279	21

group 1b, and three could not be grouped. The one English strain fitted into group 3. Of the 6 American strains five fitted into group 2c, and one could not be grouped. The 6 Belgian Congo strains failed to fit into any group.

The lysis and lysogenicity tests on the 21 ungrouped strains are recorded in Table 1. Some of these strains gave lysis patterns typical of a group but failed to show the characteristic lysogenicity. Thus strains 67, 346 and 347 gave the lysis pattern of group 3 and strain 357 gave the lysis pattern of group 1b, but all failed to show lysogenicity. Strain 393 was contaminated with phage and could not be grouped. The remaining 16 ungrouped strains gave lysis patterns different from those of the six groups. Six of these 16 strains (206 to 211) from the Belgian Congo gave identical lysis and lysogenicity patterns and will probably form a new phage group.

DISCUSSION.

The 413 strains of *S. adelaide* examined according to the phage grouping scheme of Atkinson (1955) were completely unselected and consisted of every strain of that type available in this laboratory. The scheme, with its six groups, covered all but 21 strains which failed to give reactions characteristic of any group. Among these 21 strains, however, six from the Belgian Congo gave identical reactions and appeared to form a new group, probably carrying type 3 phage. Preliminary cross-plating tests failed to reveal any mutual lysogenicity among these six strains and thus supported the suggestion that they formed a group. Another four strains, among the 21 ungrouped, showed lysis patterns typical of a group in the scheme, but failed to show the characteristic lysogenicity. This anomaly is being investigated.

Of the 392 strains giving typical group reactions, 281 came from Ceylon, of which 279 belonged to group 4 and two to group 3. No other group 4 strains were found among the remaining 111 strains from other localities. Thus without the Ceylon strains this group would not have been detected. Five strains coming from U.S.A. all belonged to group 2c, of which no other representatives were found. Group 2c was therefore considered to be an American group. The 105 Australian strains belonged mainly to group 3 in which 89 strains were placed. Group 3 was not, however, essentially an Australian group as the one English strain and two Ceylon strains belonged to it. Only Australian strains occurred in the other three groups, 1a, 1b and 2a. Thus the Australian strains covered the widest range of groups. Thirteen strains belonged to group 2a, a reasonably well represented group, but groups 1a and 1b were rare. A regional distribution of the groups was thus indicated.

SUMMARY.

A phage grouping scheme depending on lysis and lysogenicity tests was applied to 413 strains of *S. adelaide*. Three hundred and ninety-two strains gave typical group reactions and were allocated to various groups in the scheme. Twenty-one strains failed to give typical group reactions and could not be assigned to any group.

The 392 grouped strains consisted of 279 in group 4, 92 in group 3, 13 in group 2a, five in group 2c, two in group 1a and one in group 1b.

All the group 4 strains came from Ceylon, 89 of the 92 group 3 strains and all the group 1a and group 1b strains came from Australia, and all the group 2c strains from America.

A regional occurrence of these groups was thus suggested.

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ANTIBACTERIAL ACTIVITY IN MEMBERS OF THE HIGHER FUNGI

3. ANTIBACTERIAL AND ANTIPHAGE TESTS WITH PSALLIOTIN

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The recent discovery of the light sensitivity of psalliotin (Atkinson, 1954, 1955), the antibiotic of *Psalliota xanthoderma*, in crude mushroom extracts necessitated the repetition of earlier work (Atkinson, 1946, 1949) with proper protection of the antibiotic from light inactivation. The present paper records the results of repeated and extended tests on the range of action of crude psalliotin.

Materials and Methods.

The preparation of crude psalliotin was 87/54 1st extract which was also used in the light sensitivity tests (Atkinson, 1955) recorded in paper 2 of this series. 87/54 1st extract was an active extract of mushroom stems and had never been exposed to bright light.

The organisms used in the sensitivity tests were standard designated strains from type culture collections, locally isolated strains or specially imported strains (such as the Ceylon strains of *S. adelaide*) held in this laboratory for other projects. The actual strain designations are given with the results in Table 1.

The bacteriophages used in the antiphage tests were all isolated in this laboratory from lysogenic strains of *Salmonella* and, with the exception of the *S. blegdam* phage, have been described to some extent elsewhere (Atkinson *et al.*, 1952; Atkinson and Geytenbeek, 1953; Atkinson and Carter, 1953; Atkinson and Klauss, 1954).

For the antibacterial tests a large loopful of a broth culture of the test organism was streaked on a quadrant of an agar plate; and a 6 mm. disc of blotting paper, soaked in the psalliotin extract and dried at room temperature in air was placed on the inoculated area. The plates were incubated for 18 hours and occasionally for 48 hours at 37° C. The size of any inhibition zone was then noted. *S. typhi* 876, the standard test organism, was always included as a control on the activity of the discs. The medium used was beef digest agar which, for the streptococcal tests only, was enriched by the addition of 10 p.c. horse blood.

The antiphage tests were made with phage preparations diluted just to give confluent or semi-confluent lysis, which were dropped on to an area on an agar plate inoculated immediately beforehand with the host bacteria. A 6 mm. psalliotin disc was then placed on the phage drop towards the side and the plate incubated at 37° C. and examined after 18 hours. Parallel control tests of host organism and phage without the psalliotin disc, and host organism with the psalliotin disc, were done at the same time. The medium used was Oxoid nutrient broth with 5 p.c. Oxoid yeast extract, solidified with 1 p.c. agar.

All work involving the psalliotin discs or extracts was done either in the dark or in dim yellow light from a Philips yellow dark-room globe which had previously been shown not to affect the activity of crude psalliotin (Atkinson, 1954, 1955).

EXPERIMENTAL.

Antibacterial tests. These tests covered mainly Gram positive cocci and the *Enterobacteriaceae,* and numerous strains of some species were included. Thus 16 strains of *Staph. aureus* of varying antibiotic sensitivity, isolated from current infections, were tested as well as 10 standard host strains of *Staph. aureus* for the staphylococcal typing phages. The streptococci were represented by 36 strains covering the Lancefield groups A, B, C, D, F, G, H, K, L, N and O, two strains of *Strep. sanguis* and four strains of the *viridans* group. In the *Enterobacteriaceae,* 122 strains were tested of which 85 were Salmonellas (including 33 strains of *S. adelaide*), 21 belonged to the Arizona group and the remaining 16 strains represented Ballerup-Bethesda, *Klebsiella, Escherichia, Shigella* and *Proteus* groups. Three strains of *Pseudomonas* were also tested. The results of these tests are given in Table 1.

TABLE 1.

Antibacterial tests with crude psalliotin.

Test organism		Strain origin	Antibiotic zone with 6 mm. disc
Streptococci			
Group A	Type 1, SF130/13	3	++++
Group A	JAC	7	+++
Group A	Type 28, small	3	++
Group A	Type 12, SF42	3	++
Group B	R51, 457	3	−
Group B	EC29, 090	3	++
Group C	R51, 511	3	±
Group C	R51, 482	3	++
Group C	S53, 96 (*Strep. dysgalactiae*)	7	++
Group C	S53, 97 (*Strep. dysgalactiae*)	7	++
Group C	Qb (animal)	7	++
Group C	Qn (animal)	7	+++
Group C	Qw (animal)	7	++
Group D	C1	2	−
Group D	C76	2	−
Group D	CN 2626	3	±
Group D	S53, 53	7	±
Group F	S53, 31	7	+++ (after 48 hrs.)
Group G	S53, 98	7	++++
Group G	Valente	3	++
Group G	Sidebottom	3	+++
Group H	Blackburn	3	++
Group H	8684/49	3	+++
Group H	5042/48	3	++
Group K	1348/48	3	++ (after 48 hrs.)
Group K	R51, 554	3	±
Group L	SHC 176	3	++++
Group L	21203/44	3	++++
Group L	Qa	7	−
Group L	QS	7	−
Group L	QH	7	+++
Group N	Rice	3	+
Group N	R730/46	3	± (after 48 hrs.)

TABLE 1—*continued.*

Antibacterial tests with crude psalliotin.

Test organism	Strain origin	Antibiotic zone with 6 mm. disc
Group N OJ Lactis	3	++
Group O 5119/48	3	+
Group O 1360/48	3	+
Strep. sanguis 1 NCTC 7863	1	+++ (after 48 hrs.)
Strep. sanguis 1/11 NCTC 7865	1	+++ (after 48 hrs.)
viridans group S2	7	–
viridans group S3	7	–
vidirans group S12	7	++
viridans group S7	7	–
Staphylococci		
Staph. aureus NCTC 8331	1	++
Staph. aureus NCTC 8357	1	++++
Staph. aureus NCTC 8507	1	+
Staph. aureus NCTC 8369	1	+++
Staph. aureus NCTC 8509	1	++
Staph. aureus P.S. 80	7	+++
Staph. aureus NCTC 8327	1	++
Staph. aureus NCTC 8358	1	++++
Staph. aureus NCTC 8354	1	+++
Staph. aureus NCTC 8363	1	+++
Staph. aureus M1/1 (CE)	7	+++
Staph. aureus M1/2 (ASCTE)	7	+++
Staph. aureus M1/3 (PASCTE)	7	++++
Staph. aureus M1/4 (CE)	7	++++
Staph. aureus M1/5 (ASCTE)	7	+++
Staph. aureus M1/6 (E)	7	+++
Staph. aureus M1/7 (ASCTE)	7	++
Staph. aureus M1/8 (CE)	7	+++
Staph. aureus M1/9 (ASCTE)	7	+++
Staph. aureus M1/10 (ASCTE)	7	+++
Staph. aureus M1/11 (PASCTE)	7	++++
Staph. aureus M1/12 (E)	7	++++
Staph. aureus M1/13 (ASCTE)	7	++
Staph. aureus M1/14 (CE)	7	+++
Staph. aureus M1/15 (ASCTE)	7	++
Staph. aureus M1/16 (CE)	7	+++
Enterobacteriaceae		
Escherichia coli Aberdeen 1064 (O group 55)	4	++
Escherichia coli StokeW (O group 111)	4	+++
Escherichia coli Rechner (O group 111)	7	+++
Escherichia coli Edgar (O group 111)	7	+++
Escherichia coli NCTC 8196	1	–
Escherichia freundii (Schmid)	6	–
Klebsiella (*Aerobacter*) *aerogenes* NCTC 8197	1	–
Shigella (*Escherichia*) *dispar* 1	5	++++
Proteus morgani a 3915	5	+++
Proteus morgani a 1721	5	++++
Proteus morgani NCTC 235	1	+++
S. ballerup S156	5	–
Bethesda Na 11	5	±
Bethesda Md 1	5	–
Arizona 699/52	5	++

TABLE 1—*continued.*

Antibacterial tests with crude psalliotin.

Test organism	Strain origin	Antibiotic zone with 6 mm. disc
Arizona 1715/50	5	+
Arizona CDA1 44	5	++++
Arizona CDA1 125	5	++++
Arizona CDA1 434	5	++++
Arizona CDA1 184	5	–
Arizona S39	5	+++
Arizona Ore 181	5	++++
Arizona Pc 107	5	++++
Arizona Pc 145	5	++++
Arizona Pc 167	5	++++
Arizona Pc 139, S207	5	++++ (over 30mm.)
Arizona Pc 148	5	++++
Arizona Sp. 21 x 34	5	++++
Arizona NJ4	5	++++
Arizona N99	5	++++
Arizona Arizona	5	++++
Arizona Hudson	5	++++
Arizona So 5	5	++++
Arizona So 34	5	++++
Arizona So 116	5	+++
Shigella boydii 1	5	++++ (over 30mm.)
Shigella flexneri 1a	5	++++ (over 30mm.)
Salmonella aberdeen S125	1	–
S. abortus-equi S201	5	–
S. adelaide 2	7	–
S. adelaide 14	7	–
S. adelaide 20	7	–
S. adelaide 37	7	–
S. adelaide S273	5	–
S. adelaide Cey 4	6	+++
S. adelaide Cey 1921-53	6	++
S. adelaide Cey 2670-53	6	+++
S. adelaide Cey 2911-53	6	+++
S. adelaide Cey 1068-53	6	+++
S. adelaide Cey 35/28/8/53	6	–
S. adelaide Cey 39/28/8/53	6	–
S. adelaide Cey 1864-53	6	+++
S. adelaide Cey 1876-53	6	+
S. adelaide Cey 2082-53	6	+++
S. adelaide Cey 2099-53	6	+++
S. adelaide Cey 2106-53	6	++++
S. adelaide Cey 2131-53	6	++
S. adelaide Cey 2313-53	6	+++
S. adelaide Cey 2546-53	6	+
S. adelaide Cey 2367-53	6	+++
S. adelaide Cey 2391-53	6	++
S. adelaide Cey 2512-53	6	+++
S. adelaide Cey 2767-53	6	–
S. adelaide Cey 2856-53	6	++++
S. adelaide 32	7	–
S. adelaide 67	7	–
S. adelaide 191/52 Kauffmann	4	–
S. adelaide 194/52 Kauffmann	4	–
S. adelaide 362/52 Kauffmann	4	–

TABLE 1—*continued.*

Antibacterial tests with crude psalliotin.

Test organism	Strain origin	Antibiotic zone with 6 mm. disc
S. adelaide 363/52 Kauffmann	4	–
S. adelaide 364/52 Kauffmann	4	–
S. adelaide 365/52 Kauffmann	4	±
S. amager S289	4	–
S. bonariensis S173	5	–
S. berta S146	8	–
S. bredeney S290	4	–
S. blegdam Kidd	7	–
S. bovis-morbificans 8	7	–
S. bovis-morbificans 13	7	–
S. bovis-morbificans 14	7	–
S. bovis-morbificans 17	7	–
S. bovis-morbificans 18	7	–
S. cerro S138	8	–
S. cholerae-suis S24	1	++++
S. cholerae-suis var. *kunzendorf* S25	1	+++
S. derby S9	2	–
S. dar-es-salaam S44	1	–
S. dublin S124	1	–
S. enteritides S32	1	–
S. gallinarum S41	1	++
S. hvittingfoss S133	8	–
S. london S120	1	–
S. muenchen S39	1	–
S. moscow S145	8	–
S. monschaui S280	5	–
S. newport S45	1	–
S. orion S292	4	–
S. potsdam Churcher	7	–
S. potsdam S38	2	–
S. potsdam 1024A	7	–
S. potsdam 1024B	7	–
S. paratyphi A S293	4	–
S. paratyphi B S5	1	++++
S. pullorum S223	5	++++ (over 30mm.)
S. poona S116	8	–
S. reading S56	2	–
S. rostock S15	1	–
S. san-diego S199	5	–
S. senftenberg 1	7	–
S. senftenberg 20	7	–
S. senftenberg 36	7	–
S. senftenberg 60	7	–
S. senftenberg 63	7	–
S. senftenberg 68	7	–
S. senftenberg 70	7	–
S. typhi S76	7	+++
S. typhi H901 S93	1	++++
S. typhi 0901 S49	9	++++
S. typhi-murium S175	5	–
S. waycross S215	5	–
S. waycross Cronin	7	–
S. waycross Currie	7	–

TABLE 1—*continued.*

Antibacterial tests with crude psalliotin.

Test organism	Strain origin	Antibiotic zone with 6 m.m. disc
Pseudomonadaceae		
Pseudomonas aeruginosa NCTC 6750	1	—
Pseudomonas aeruginosa T5	7	—
Pseudomonas aeruginosa T8	7	—

— = no antiboitic zone.
± = very small zone, average diameter, less than 10 mm., or partial inhibition only.
+ = average diameter 10-14 mm.
++ = average diameter 14-18 mm.
+++ = average diameter 18-22 mm.
++++ = average diameter greater than 22 mm.

The capital letters (in brackets) following the strain numbers of the local strains of *Staph. aureus* M1/1-M1/16 indicate their antibiotic sensitivities as follows:—
P = sensitive to penicillin.
A = sensitive to aureomycin.
S = sensitive to streptomycin.
C = sensitive to chloramphenicol.
T = sensitive to terramycin.
E = sensitive to erythromycin.

The sensitivities were determined by the paper disc method according to Mackie and McCartney (1953).

Tests with streptococci were made on beef digest agar with 10% horse blood, other tests on beef digest agar.

The figures under "strain origin" indicate the following sources:
1. National Collection of Type Cultures, London.
2. Commonwealth Serum Laboratories, Melbourne.
3. Streptococcal Reference Laboratory, London.
4. International Enterobacteriaceae Centre, Copenhagen.
5. Communicable Diseases Centre, Atlanta.
6. Institute of Medical Research, Colombo.
7. Locally isolated.
8. Veterinary Research Station, Glenfield.
9. C.S.I.R.O.

Of the 42 strains of streptococci tested, 29 were unmistakably inhibited by the psalliotin discs (+ to ++++), five showed very small inhibition zones or zones of reduced growth (±) and eight were unaffected (—). The 29 sensitive strains consisted of all strains tested of Group A (4 strains), Group F (1 strain), Group G (3 strains), Group H (3 strains), Group O (2 strains) and *Strep. sanguis* (2 strains) as well as one out of two Group B strains, six out of seven Group C strains, one out of two Group K strains, three out of five Group L strains, two out of three Group N strains, and one out of four *viridans* group strains. The five strains showing slight inhibition or reduced growth consisted of one Group C, two Group D, one Group K and one Group N. Thus all strains of Groups A, C, F, G, H, K, N, O and *Strep sanguis* were inhibited to some extent

by the psalliotin discs. In Groups B and L sensitive and resistant strains occurred, Group D strains were resistant or only slightly affected, and three out of four of the *viridans* group were also resistant. All the 26 strains of staphylococci tested were sensitive to psalliotin irrespective of their sensitivity to other antibiotics which ranged from sensitivity to all six test antibiotics (penicillin, aureomycin, streptomycin, chloramphenicol, terramycin, erythromycin) to sensitivity to erythromycin only (strains M1/6 and M1/12).

The results with the *Enterobacteriaceae* were extremely varied, some members being resistant, others providing the most sensitive organisms so far found. Variations occurred not only among species but also among types and even strains. Of the six strains of *Escherichia* tested, four strains of *E. coli* of O groups 55 and 111 were sensitive, as also was one strain of *Shigella dispar* which, according to Kauffmann (1951), should be placed with the *Escherichia*. However, the other two strains of *Escherichia* were resistant. All strains of *Proteus morgani* were sensitive, but the Ballerup-Bethesda strains were resistant. The Arizona group, however, provided many very sensitive strains and out of 21 tested only one (CDA1 184) was resistant. Most of the others gave very good ones of inhibition (++++) and strain Pc 139 S207 showed one of the largest zones (over 30 mm.) seen in this work. The two *Shigella* strains tested were also very sensitive and gave large zones over 30 mm. in diameter. In the Salmonella group 35 types, mainly represented by single strains, were tested. Of these, five types, *S. cholerae-suis* (2 strains including one *kunzendorf*), *S. gallinarum* (1 strain), *S. paratyphi* B (1 strain), *S. pullorum* (1 strain), and *S. typhi* (3 strains) were sensitive, and *S. pullorum* S223 gave a very large zone over 30 mm. in diameter. *S. adelaide*, of which six strains (2, 14, 20, 37, S273 and Cey4), representing phage groups suggested by Atkinson and Geytenbeek (1953) and Atkinson and Klauss (1954), were first tested, presented the rather curious result that strain Cey4 (from Ceylon) was sensitive and the others were resistant. Therefore 19 more representative Ceylon strains of *S. adelaide* (Cey 1921-53 to Cey 2856-53 in Table 1) were tested with psalliotin discs. Of these 19 strains, 16 strains resembling the type strain Cey4 were sensitive, two strains (35/28/8/53 and 39/28/8/53) resembling the type strain 14 were resistant, and one strain (2767-53), which failed to fit into any of the suggested phage groups, was also resistant. Thus all the Ceylon strains of the Cey4 type tested here resembled Cey4 in their sensitivity to psalliotin. Ceylon strains not like Cey4 were resistant. Eight further strains of *S. adelaide* which could not be allotted to any phage group were also tested and found resistant. Six of these (191/52 to 365/52 in Table 1) came from the Belgian Congo, and the other two strains (32 and 67) were locally isolated. Thus the 33 strains of *S. adelaide* could be divided into psalliotin sensitive (17 strains) and psalliotin resistant (16 strains), the sensitive strains all belonging to the same phage group as Cey4, the resistant strains including all other strains tested.

On account of this variation in psalliotin sensitivity among strains of *S. adelaide* of different phage groups, other Salmonella types, for which phage groups had been established or were under investigation in this laboratory, were further tested. Thus 5 strains of *S. bovis-morbificans* (8, 13, 14, 17 and 18) of different phage groups suggested by Atkinson *et al.* (1952), 4 strains of *S. potsdam* (unpublished phage groups represented by Churcher, S38, 1024A and 1024B), 6 strains of *S. senftenberg* (unpublished phage groups represented by 1, 20, 36, 60, 63, 68 and 70) and 3 strains of *S. waycross* (S215, Cronin and Currie) of two different phage groups suggested by Atkinson and Carter (1953) were selected. All these strains were resistant to the psalliotin discs.

The three strains of *Pseudomonas aeruginosa* were unaffected by the psalliotin discs.

Antiphage tests. The results of these tests with some Salmonella phages isolated in this laboratory are given in Table 2.

TABLE 2.

Antiphage tests with crude psalliotin.

Test phage	Host organism	Results	
		With Psalliotin 6 mm. disc	Without Psalliotin disc
S. adelaide phages			
Ad2	*S. adelaide* 14	CL	CL
Ad14	*S. potsdam* Churcher	CL	CL
Ad20	*S. adelaide* 14	CL	CL
Ad37C	*S. adelaide* 14	CL	CL
Ad37H	*S. adelaide* 14	CL	CL
Ad273	*S. adelaide* 14	CL	CL
S. blegdam phage Huchinson	*S. blegdam* Kidd	CL	CL
S. waycross phage W Cr	*S. waycross* Currie	CL	CL
S. bovis-morbificans phages			
BM 7H	*S. bovis-morbificans* 18	CL	CL
BM 7C	*S. bovis-morbificans* 18	SCL	SCL
BM 14	*S. bovis-morbificans* 18	CL	CL
BM 45	*S. bovis-morbificans* 18	SCL	SCL

CL = Confluent lysis.
SCL = Semi-confluent lysis.

No inhibition of phage action by the psalliotin discs was observed in these tests, which included phages of different plaque type, heat resistance and serological reaction representative of the Salmonella phages so far characterized here. The host organisms used for growth of the phage were unaffected by the psalliotin

discs. For cultivation of the *S. adelaide* phage Ad14, for which the routine host strain *S. adelaide* Cey4 was sensitive to psalliotin, the resistant strain *S. potsdam* Churcher was used.

DISCUSSION.

Obviously the tests reported here were restricted by the limited concentration of psalliotin in the available crude extracts. Higher concentrations of psalliotin might be effective against some of the species described as resistant. Nevertheless, the most sensitive species and strains were detected in the present tests and most of them gave clear-cut results. A few strains, however, showed only very small zones or zones of reduced growth (±). They would probably be sensitive to stronger concentrations of psalliotin as similar very small zones or zones of reduced growth could be produced with sensitive strains by reducing the concentration of psalliotin on the test disc. Most of the streptococci were sensitive, the main exceptions occurring in the *viridans* group and Group D, some of which gave a ± reaction and therefore were not completely resistant. The staphylococci were all sensitive irrespective of their reactions to the six commonly used antibiotics. Thus no cross resistance was detected between any of these antibiotics and psalliotin.

In the *Enterobacteriaceae*, among the numerous Salmonella types tested only a few were sensitive to psalliotin and *S. adelaide* showed a peculiar strain variation in which the group of Ceylon strains resembling strain Cey4 were sensitive and strains of all other groups were resistant. The strains of the Cey4 group were characterized by Atkinson and Klauss (1954) on their ready lysis with all the *S. adelaide* type phages and their failure to reveal any *S. adelaide* phages in lysogenicity tests. Most of the other strains of *S. adelaide* included in the psalliotin tests and found resistant were lysogenic carrying phage Ad14 alone or with other phages. Carriage of phage Ad14 might therefore possibly be related to resistance to psalliotin since Stocker, Zinder and Lederberg (1953) have shown transduction of serological characters in Salmonella cultures by phage. Work is therefore proceeding on the treatment of strain Cey4 and related strains with phage Ad14 followed by testing for psalliotin resistance. The other Salmonella types for which strains of various phage groups were tested (*S. bovis-morbificans, S. potsdam, S. senftenberg* and *S. waycross*) were all resistant to psalliotin. Obviously, however, sensitive strains of apparently resistant types might be found, as with *S. adelaide*, by examination of a sufficient range of varieties of each type.

Psalliotin, in the available concentration, showed no activity against various Salmonella phages. Other phages must be tested, however, with the psalliotin and might prove sensitive, as Asheshov *et al.* (1954) found great variation in sensitivity of a wide range of phages to various actinomyces antibiotics.

The antibiotic spectrum of psalliotin in its present crude and unconcentrated form appears to be reasonably wide covering many Gram positive cocci and a number of Gram negative intestinal pathogens.

SUMMARY.

Forty-two strains of streptococci of various groups, 26 strains of *Staph. aureus* of varying antibiotic sensitivities, 122 strains of the *Enterobacteriaceae* and 12 types of Salmonella bacteriophage were tested by a paper disc method for sensitivity to a crude extract of the light-sensitive antibiotic psalliotin. Streptococci of groups A, C, F, G, H, K, N and O, *Strep. sanguis* and all strains of *Staph. aureus* were sensitive to the psalliotin extract. Among the *Enterobacteriaceae* sensitive strains occurred in the *Escherichia* O groups 55 and 111, the Arizona group, the *Shigella* group and *Proteus morgani*. Of 35 Salmonella types tested, five, *S. cholerae-suis*, *S. gallinarum*, *S. paratyphi* B, *S. pullorum* and *S. typhi* were sensitive. *S. adelaide* strains from Ceylon were also sensitive. Other *S. adelaide* strains were resistant. None of the 12 Salmonella bacteriophages were obviously affected by the psalliotin extract.

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AN ANALYSIS OF SOME FACTORS AFFECTING EXPERIMENTAL ASCITES IN THE RAT

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Experimental ascites in animals is known to be difficult to produce and maintain. The commonest method used has been supradiaphragmatic constriction of the inferior vena cava. In the rat, however, this has not been successful (Volwiler, Grindlay and Bollman, 1950; Bollman, 1953; Lake, 1953); nor has gross ascites in the rat resulted in our experiments from a variety of other methods, some reported successful in other species. One of these, partial occlusion of the portal vein, does give small amounts of ascites in the first few days, but this is no longer evident after one week (Kunkel and Eisenmenger, 1949).

The difficulty in obtaining gross ascites in the rat might be due in part to a large reserve of the absorptive mechanism from the peritoneal cavity (*cf.* Courtice and Simmonds, 1954). It was, therefore, decided to study some of the factors aiding the absorptive capacity from the peritoneal cavity in normal rats and to ascertain any change in this function following partial constriction of the portal vein. When it was found that the maximum absorptive capacity was very high in both normal and ascitic rats, other factors likely to precipitate massive ascites were employed. In particular, massive intravenous (I-V) infusions of Ringer-Locke solution were investigated.

METHODS.

Rats used were between 150 and 325 gm. weight, the majority male. Partial occlusion of the portal vein was achieved by a cotton thread (Reynell, 1952). This always produced visible engorgement of gut veins with cyanosis of the gut wall. The mortality rate was 40 p.c. The recovered animals were studied in groups mostly at 24 hours, 4 days and 1 month after the ties were made. Sham-operated and unoperated rats were used as controls, no difference being observed in these groups. Free access to food and water was allowed, unless otherwise noted. The rats were studied unanaesthetized, unless otherwise noted. All rats were autopsied.

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Absorption rates from the peritoneal cavity were studied by means of protein labelled with the dye, T1824, rat plasma diluted with an equal volume of Ringer-Locke solution. For details of the injections see Courtice and Steinbeck, 1950a and b; 1951a. Maximal absorption was determined following a continuous intraperitoneal (I-P) infusion over 24 hours of 0·9 gm. p.c. bovine albumin in Ringer-Locke solution delivered through a transflex catheter that had been inserted into the peritoneal cavity aseptically. The drip rates were checked regularly and the flow was maintained so that fairly large amounts of fluid were present in the cavity throughout. Maximum absorption was then calculated by the difference in amount delivered and that remaining at the end of the experiment.

I-V infusions of Ringer-Locke solution were carried out over 5 hours through a polyethylene tube in the right external jugular vein. The solution consisted of Na 154 meq./l., K 5·4 meq./l., Ca 2·8 meq./l., Cl 164 meq./l., HCO_3 2·4 meq./l. and glucose 1 gm./l. used freshly made up or after sterilization.

Portal pressures. A hypodermic needle, with side perforations near the bevel, attached to a simple saline manometer was inserted into the lower end of the superior mesenteric vein and the pressure read at equilibration. The system conformed to the rats' size. In all cases, the pressure was read with the rat under as light ether anaesthesia as possible, since deep ether or "nembutal" caused a considerable fall in pressure. The abdomen was left open with the animal supine (*cf.* Wiggers, Opdyke and Johnson, 1946). Slight bleeding around the needle also caused a prompt and maintained fall of venous pressure, and where this occurred the reading was discarded. Simple insertion was found to be better than a tied-in indwelling catheter because of damage to vessels and nerves and delayed infarction of the gut behind the insertion. Length of time under anaesthesia was often important; a sustained pressure fall in some rats was observed after one quarter hour.

Nitrogen was determined in plasma and serous fluids by a micro-Kjeldahl method. Globulins were precipitated by 21 p.c. sodium sulphite (Campbell and Hanna, 1937). NPN corrections were always made from calculations based on trichloracetic acid filtrates of the sample fluids.

T-1824 dye was extracted by the method of Harington, Pochin and Squire (1940) and the concentrations read in a Hilger "Spekker" absorptiometer.

Sodium and potassium were determined on the flame photometer attachment of a Beckman DU spectrophotometer.

Chloride was determined by the method of Schales and Schales (1941) and bicarbonate by the titrimetric method of Van Slyke, Stillman and Cullen (1919). Urinary titratable acidity was read by a modification of this method. For these determinations the samples were collected under paraffin and read immediately at the end of the experiments.

Plasma volume changes following the I-V infusions were measured as a p.c. ratio of haemoglobin concentration difference before and after transfusion over initial concentration, the haemolysed blood samples being read in a calibrated MRC grey-wedge photometer.

Organ weights were recorded at autopsy.

Statistical analyses were used throughout, as detailed by Mather (1949).

RESULTS.

Fig. 1 shows the ascites formed after partial occlusion of the portal vein. It was maximal for the first two days and dropped to a low but abnormal amount for the next week and there was no free fluid after a month. The protein concentration of pooled fluids at these times was always high, ranging from 3·05-3·34 gm. p.c. In addition to the free fluid, marked oedema between the leaves of the mesentery was observed, especially during the first 48 hours.

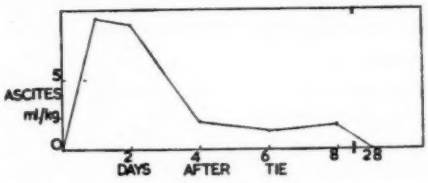


Fig. 1. The ascites formed after partial occlusion of the portal vein in rats. Average of groups of 10.

Absorption studies.

To ascertain whether absorption was proceeding normally along the lymphatics, a small amount (4 ml./kg.) of dye-labelled protein (4 mg./kg. T1824) was injected into the peritoneal cavity of normal and 24-hour portal tied rats. The plasma dye levels were determined 1 and 3 hours later (Fig. 2a).

As these levels did not differ significantly at either time, this suggested that the rate of protein absorption was at least as fast as normal in the 24-hour tied rats; it was probably faster, as the injected dye-protein would presumably have been diluted with preformed ascites in this group, but by what margin it was not possible to detect.

It was noted that approximately the injected amount had disappeared in the tied group after one hour with little difference thereafter. These results indicated that there was no blockage to absorption by the lymphatics, and

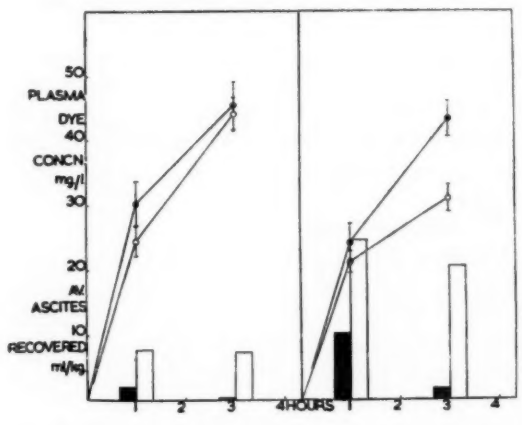


Fig. 2. The plasma dye levels and abdominal free fluid present after the I-P injection of 4 ml./kg. and 20 ml./kg. of dye-labelled protein-fluid in rats. Groups of 6.

L.H.S. (a)=after 4 ml./kg. I.P.I.
R.H.S. (b)=after 20 ml./kg. I.P.I.
●=normal.
○=24-hour portal vein tied rats.
I =standard deviations.

that injection had stimulated increased absorption of free fluid in the tied group. Free fluid therefore might be present in the peritoneum without implying maximal absorption or lymphatic blockage.

A larger volume of protein-fluid (20 mg./kg.), labelled as before, was then introduced into the peritoneal cavities of either group. Under these conditions it was seen (Fig. 2b) that the plasma dye levels, abreast at 1 hour, were significantly different at 3 hours ($P<0{\cdot}01$), being higher in normals. Here dilution of the dye-label would have been less obvious and volume carriage more important than after the 4 ml./kg. injection in the portal tied rats (Fig. 1). The added fluid was reduced in quantity in both groups but at an apparently slower rate in the portal tied rats. These facts showed that absorption rates did not differ among rats with or without ascites if the volumes to be absorbed were small. If the extra volume of fluid was large, however, the portal tied rats, who were already absorbing at a high level prior to injection, could only cope with a correspondingly smaller fraction of the new load compared with normals, and remove it from the peritoneum at an apparently slower rate.

When continuous production of ascites was simulated by 24 hours I-P infusion the figures for maximal absorption over this period were actually $292\pm28{\cdot}0$ ml./kg. and $156\pm3{\cdot}2$ ml./kg. in groups of 4 rats each, normal and 24-hour portal vein tied respectively. It was clear, therefore, that in the tied rats, much less infused fluid was required to reach the maximum absorptive capacity.

It was apparent from these three sets of I-P injection experiments that up to a maximum limit, ascites was absorbed at a rate proportional to its volume, i.e., the more fluid present the quicker it was absorbed. This applied to both normals and portal tied rats, but reached an apparently lower limit of about 50 p.c. normal in the latter due to these rats' own production of ascites.

Effect of massive Ringer-Locke I-V infusions.

This effect was studied in two ways, both of them entailing a steady rate transfusion over 5 hours. In the first, a load range of over 500 ml./kg./5 hours was studied in normal and portal vein tied rats. By this means certain facts were made apparent and these were tested and confirmed in the second series, where a fixed load of 300 ml./kg./5 hours was given each rat studied. Fig. 3 and Table 1 embody the fluid data for these experiments.

In all groups, only a little fluid accumulated in the peritoneal cavity, always more in the pleural cavities, and there was always massive oedema of the intramesenteric and retroperitoneal areas. This oedema was more striking than that in the simple portal vein ties already mentioned. In normals the amount of ascites was proportional to the oedema load ($P<0{\cdot}001$), but there was no such simple relation in the portal tied groups.

The portal tied groups deserve separate comment. The 4-day group had

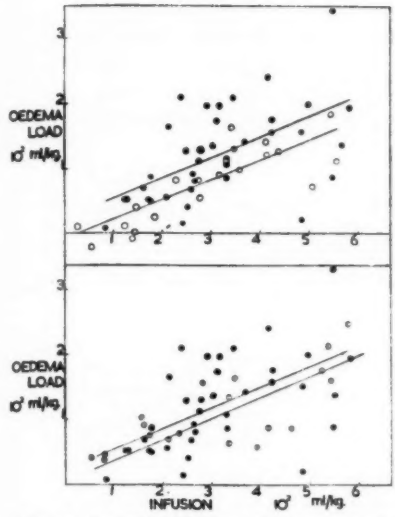
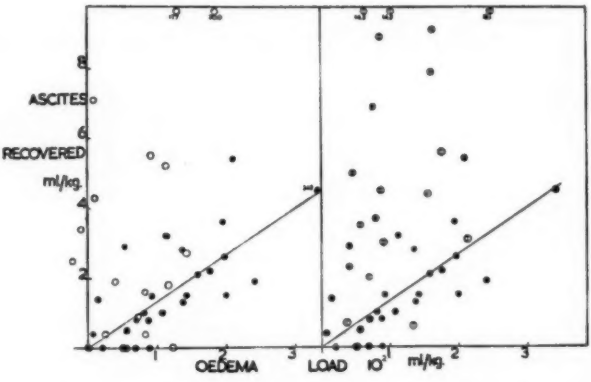


Fig. 3. (a) The relation of oedema load to infusion in rats following Ringer-Locke solution I.V.I. over 5 hours. The lines drawn are calculated regression lines and are all significant ($P<0.001$).



(b) The relation of ascites recovered to oedema load in rats following the above infusions. The line drawn for normals is the calculated regression line and is significant ($P<0.001$).

●=normal.
○=portal vein part occluded 4 days.
⊙=portal vein part occluded one month.

TABLE 1.

The Effect on Fluid Balance of 300ml./kg./5 hrs. of Ringer-Locke Solution I-V. Groups of 6 Rats Av.+S.E.

Measured ml./kg.	Normals	Portal vein tie 4 days	Portal vein tie 1 month
Oedema load	158±23	62±19	167±21
Ascites	1·0±0·4	5·0±1·7	7·6±2·0
Pleural Fluid	10·8±4·3	9·3±4·6	21·8±3·9
Urine	149±27	246±15	140±27

no significantly greater amount of ascites after transfusion than the normals, but this might well have been due to the fact that for both the range and the comparable dose this group always retained much less fluid. The one-month group, whose fluid retention matched normal, were, however, liable to have larger serous effusions, peritoneal and pleural, on both experiments.

Analysis of some likely operative factors was now carried out.

Portal pressures and proteins. Provided that there is no alteration in capillary permeability, the production of excess tissue fluid will be determined

by the positive balance between capillary pressure and the osmotic pressure of the plasma proteins. Portal vein pressure gives some lower indication of draining capillary pressures, and the albumin level of the osmotic pressure. The pressure changes resulting from portal tie and at the end of the 5-hour transfusion are shown in Table 2. It was evident that the pressure was significantly

TABLE 2.

Portal Pressures and Plasma Proteins in Rats with and without 5 hour I-V. Ringer-Locke Infusions.
Portal Pressure Group Nos. = 4-8
Protein Group Nos. = 6-20. *Av. and S.E.*

Type	No infusion				
	Portal pressure cm. saline	Plasma albumin gm.p.c.	Total plasma protein gm.p.c.	Calc. C.O.P. cm. H_2O	Av. change in filtration pressure cm. H_2O
Normal	14·1±1·4	3·67±0·06	6·15±0·12	26·4	
Portal vein tie 4 days	24·8±1·8	3·32±0·03	5·51±0·18	22·7	14·4
Portal vein tie 1 month	18·9±0·9	3·77±0·01	6·27±0·33	27·3	3·9

Type	With infusion				
	Portal pressure cm. saline	Plasma albumin gm.p.c.	Total plasma protein gm.p.c.	Calc. C.O.P. cm. H_2O	Av. change in filtration pressure cm. H_2O
Normal	22·6±1·8	2·91±0·18	4·66±0·14	18·0	
Portal vein tie 4 days	26·6±2·5	2·70±0·56	4·76±0·34	17·8	4·2
Portal vein tie 1 month	22·0±1·7	2·92±0·20	4·77±0·28	18·4	−1·0

increased by both these procedures. The values for plasma proteins are also shown in Table 2. There was a small but significant fall in the protein fractions in the 4-day group.

Calculation of the osmotic pressures by the method of Wells, Youmans and Miller (1933) when compared with the estimate of capillary pressure based on the venous figures, shows that there is an average net increase in filtration pressure of about 14 cm. water 4 days after portal tie and of about 4 cm. water 1 month after constriction. These figures, then, suggested that ascites would be more likely to form in the 4-day group, yet, despite the high rise in filtration pressure, there was little ascites at 4 days.

In regard to the drop in protein concentrations on transfusion, it was noted in all animals that the albumin/total ratio always approximated 60 p.c., as was

TABLE 3.

Electrolyte Data following 300 ml./kg./5 hrs. Ringer-Locke I-V. Groups of 6 Rats. Avs. + S.E.

	Concentrations meq./l.*				
	Na	K	Cl	HCO_3 Titrac. Ac.	N mg.p.c.
Normal	141·7±2·9 154·5±9·9	8·5±0·69 16·6±1·26	119·2±3·8 170·8±10·3	19·9±1·4 +0·32±0·65	412±49·7
Portal vein tie 4 days	142·5±1·6 133·3±1·4	6·8±0·47 15·4±1·23	117·3±2·1 149·0±3·3	25·3±1·6 −4·64±0·81	230±6·3
Portal vein tie 1 month	142·3±2·0 159·0±16·4	7·07±0·67 23·2±2·25	127·0±3·2 181·0±16·4	21·4±0·9 +3·35±1·77	423±20·1

	Amounts Excreted meq./kg.				
	Na	K	Cl	Titrac. Ac.	N mg./kg.
Normal	22·1±3·5	2·58±0·56	24·6±3·8	−0·06±0·13	552±58
Portal vein tie 4 days	32·6±2·1	3·72±0·18	36·8±2·2	−1·13±0·21	568±39
Portal vein tie 1 month	21·3±4·0	3·0±0·52	24·3±4·4	+0·29±0·11	553±83

* Top line = final plasma concns. Bottom line = urinary concns.

the case prior to transfusion, so that these changes were occasioned by simple dilution. Determinations on the serous fluid, resulting from transfusion, showed that the protein content was high and that the albumin/total ratio was raised to more than 70 p.c. In a typical case, the total protein and albumin plasma levels were 4·53 and 2·83 gm. p.c. respectively with ascitic fluid levels 3·38 and 1·95 gm. p.c. respectively. Ascitic and pleural fluids were always of the same order concentration. This meant that despite a fall in plasma osmotic pressure on transfusion, the tissue fluid protein had remained at presumably constant level (*cf.* protein in ascites with a simple tie in the portal vein) and seemingly this would have enhanced the outward movement of fluid from capillaries to tissues.

Plasma volume changes remained unrelated to the oedema load and did not differ significantly among the groups. For the range data of Fig. 3a and b, these increases were (average+S.E.) 23·8±3·9 p.c., 26·0±2·6 p.c. and 29·4±3·3 p.c. for normal, portal tie 4-day and 1-month groups respectively. The drop in

average albumin concentration on transfusion for these respective groups (Table 2), involving a different set of rats, was 20·7 p.c., 18·7 p.c. and 22·5 p.c. This also suggested that the drop in plasma albumin on transfusion had mainly been brought about by simple dilution. As the turnover of albumin on massive transfusion is faster than that of the globulin group and equilibrium more speedily achieved, comparison of the albumin levels before and after acute massive infusion is a more valid indication of change than that in total protein or globulin levels.

So far, the findings had seemed to point to the occurrence of more free fluid in the 4-day portal tie group than in the 1-month tie group, but this had not happened. The oedema load levels appeared then to be of even greater significance. As it was likely that the handling of electrolytes, and so the handling of fluid, was different among the groups, these were next studied. Urinary N was also estimated to cover urea and ammonia, the other major osmotic components of the urine.

Electrolytes. Table 1 has presented the fluid balance data and Table 3 shows the electrolyte data following Ringer-Locke 300 ml./kg./5 hours I-V. The final plasma concentrations were alike except for the significantly different normal bicarbonate level in the 4-day group compared with the low control and 1-month tie levels.

The urinary concentrations showed that sodium was excreted at around the concentration delivered and that potassium and chloride were excreted at greater concentration, the potassium concentration being three to four times that delivered. It was higher in the 1-month group than in normals. In all rats the chloride concentration was an exact balance of that of the sodium plus potassium. The 4-day group had a lower average level for each of the measured electrolytes, especially in regard to nitrogen and to titratable acid, where the urine was alkaline.

Absolute amounts excreted are of interest for all groups excreted similar amounts of nitrogen. The 4-day group, however, achieved this along with a greater output of sodium and chloride than normals.

To study whether these effects were liable to occur under different conditions, especially where the plasma-tissue protein pool was not under such severe stress, the electrolytes were studied in the same three groups, normal, 4-day and 1-month portal tie by means of an I-P infusion of 0·9 gm. p.c. albumin in Ringer-Locke solution over 24 hours. It will be recalled that the 4-day group had shown lowered total protein and albumin concentrations initially. The technique of these experiments was as for maximum peritoneal absorption, save that the rats were not allowed food and water in the experimental period. Minimal endogenous electrolyte excretion would be expected as the rats were unfed, and minimal metabolic disturbance in view of the parental glucose and albumin. In any case the urinary volumes were many times larger than under

normal conditions. Absolute excretions could not be compared, as the 1-month group surprisingly absorbed smaller amounts than the normal and 4-day group (Table 4).

TABLE 4.

Water and Electrolyte Data in Rats following 0·9 p.c. Bov. Albumin Ringer-Locke infusion I-P. for 24 hrs.

Groups of 6. No food or water. Av. + S.E.

Type and 24 hr. Absorption ml./kg.	Concentrations meq./l.*				
	Na	K	Cl	HCO-Tit. Ac.	Tot. Prot. gm.p.c. N mg.p.c.
Normal 367±27	141·8±2·7 139·0±2·5	6·7±0·48 27·8±1·4	107·8±2·4 164·3±2·9	23·6±1·2 6·95±0·85	7·31±0·35 583±28
Portal vein tie 4 days 361±19	136·5±0·8 128·3±3·1	8·22±0·45 21·8±2·8	106·0±1·1 162·5±4·1	26·4±1·1 1·57±3·32	6·24±0·15 417±27
Portal vein tie 1 month 242±28	139·2±1·5 149·8±6·5	6·22±0·45 34·7±4·2	105·8±4·7 198·5±11·5	28·2±0·6 7·77±1·79	8·1±0·61 785±90

* Top line = final plasma concn. Bottom line = urinary concn.

Type and 24 hr. Absorption ml./kg.	p.c. Amount $\frac{\text{Excreted}}{\text{Absorbed}}$				
	Na	K	Cl.	N mg./kg.	Fluid
Normal 367±27	55·8±6·5	324±39	70·0±9·6	36·4±6·9	63·8±8·4
Portal vein tie 4 days 361±19	73·6±4·2	352±19	82·8±2·7	25·4±2·0	88·2±4·1
Portal vein tie 1 month 242±28	41·6±6·5	279±76	68·2±10·3	24·0±5·0	42·8±6·0

Ratios, therefore, of amount excreted/amount absorbed are presented, for once the fluid was absorbed the rats were obliged to deal with it as a circulatory load.

As might be expected from the different conditions of loading in the two groups of experiments, some differences existed. These were, no doubt, reflections of the protein loading—for all final plasma levels were significantly raised above resting levels, with the 4-day group still the lowest—and of the gentler and longer acting nature of the I-P experiments. In spite of these differences,

however, plasma electrolyte levels in the I-P experiments were similar among the three groups save for the significantly higher K level in the 4-day group. The urinary concentrations did not differ significantly between normals and 1-month groups in any measurement, but the 4-day group had significantly lower concentrations in all these save titratable acid. Further, per cent. excretion of fluid absorbed was increased significantly in this group, as was also sodium and chloride. As some indication of minimal intergroup metabolic interference might be mentioned the similar p.c. excretion of absorbed K and absorbed albumin N in the groups. Throughout both types of experiment the variation in electrolyte levels was always largest in the normals, slightly less so in the 1-month group and minimal in the 4-day group.

These two sets of complementary electrolyte data therefore explained some of the differences observed in regard to oedema load on transfusion, namely, that 4 days after portal tie, rats in whom spontaneous ascites formation was falling to a low level were unable to retain Ringer-Locke because it induced a marked saline diuresis in them, saving them from the increase in tissue fluid production that must underlie ascites.

So far then, the analysis of likely operative factors in the production of ascites after transfusion had shown in normal rats that great as the maximum absorptive capacity of the peritoneum might be, this limit could be reached with massive Ringer-Locke infusions, which induced a marked rise in effective portal filtration pressure by virtue of raising the portal venous pressure and reducing the colloid osmotic pressure, along with a gross increase in body fluid and electrolytes.

In the 4-day portal tie group, the importance of oedema load was manifest. For despite a higher effective filtration pressure with higher venous pressure and lower colloid osmotic pressure than in normals and equivalent peritoneal absorptive capacity, ascites failed to form in quantity significantly greater than in normals after transfusion. This was apparently attributable to the markedly smaller oedema load in this group.

In the 1-month group, the factor of oedema load, equivalent to normal, coupled with a lowered peritoneal absorptive capacity was sufficient to counteract a small decrease in filtration pressure and produced serous effusions of significantly greater volume than in normals, following transfusion.

A further distinguishing feature in the handling of the infused fluid, and consequently in the production of ascites, between the normal–1-month portal tie groups on the one hand, and the 4-day portal tie group on the other, was the inability of the 4-day group to sustain equivalent albumin and total protein levels under resting or experimental conditions. The initial plasma volumes were probably of the same order, as the average pre-I-V infusion haemoglobin levels were 14·9 gm. p.c. and 15·0 gm. p.c. in normal and 4-day tie groups, so that the lowered protein levels (in the latter) did not simply betoken enlargement of plasma volume. This made the explanation unlikely that maximum distensibility

of the plasma volume was reached more quickly and hence Ringer-Locke excreted more readily on transfusion in the 4-day group.

Finally, two sets of observations are mentioned on which more detailed work is being carried out.

The effects of feeding.

Until the last mentioned I-P experiments, all work had been done in rats allowed free access to food and water until used, and in the first series of 24-hour absorptions while the experiment was proceeding. The effects of feeding the standard rat-nuts plus water on the absorption of albumin-Ringer-Locke solution are shown below. An analysis of variance showed that feeding had a very marked effect on absorption as well as portal vein constriction, but there was no significant effect from their interaction.

	Fed	Unfed
Normal	292±28·0	354±25·9
24-hour portal ties	156±3·2	302±26·9

(Figures in ml./kg., 4 rats to a group.)

Thus maximal peritoneal absorption is not a simple fixed limit in the rat but may depend upon such factors as access to food and water, especially in the presence of acute portal obstruction. Notwithstanding this, the range does not vary nearly so widely in the combined unfed, unwatered normal and portal tied series.

Normals	Portal vein tie		
	1-day	4-day	1-month
367±27	302±27	361±19	242±28

Portal pressure in the dog is known to be raised by feeding (Volwiler, Grindlay and Bollman, 1950) and this is also true in normal rats (groups of 5):

Treatment	Pressure cm. saline
Starved 24 hrs., water *ad lib.*	11·4±1·0
Food+3 hrs.	14·3±0·6
Food *ad lib.* to 5 hrs. before	14·2±1·1

The simple portal pressures of Table 2 are those of groups of rats 5 hours removed from food. At their absolute value, there is no obvious comparison with maximal peritoneal absorption in the comparably treated unfed rats. However, one of the possibilities being investigated is that *differences* in filtration pressure in the portal system affecting the production of tissue fluid and hence its removal by a final common pathway might allow more or less fluid to be absorbed from the peritoneum. According to Abdou, Reinhardt and Tarver (1952), the thoracic duct in the rat drains both peritoneal and mesenteric areas, and it might be expected that a reciprocal relationship in regard to maximal lymph carriage exists between the two.

The effect of portal tie and infusion on organ weights.

As a simple guide to the effects of portal vein tie, liver, spleen and gut+content weights were followed. The last showed no change. However, the liver and spleen did so, as shown below. The effects on these of 5-hour I-V infusion of Ringer-Locke in the normal and portal tied rats reported above are also shown.

	Liver gm./kg. Av. and S.E.		Spleen gm./kg. Av. and S.E.	
	Before infusion	After infusion	Before infusion	After infusion
Normal	35·6±0·7 (10)	43·4±1·2 (27)	2·3±0·3	3·9±0·4
Portal vein tie 4 days	42·8±2·7 (10)	36·3±1·0 (17)	7·3±0·8	5·5±0·3
Portal vein tie 1 month	46·0±0·4 (10)	36·7±1·0 (18)	4·4±0·6	4·0±0·1

Parentheses=No. in groups for both liver and spleen weights.

As these changes may occur so rapidly, it is likely that fluid shift within the liver is involved. Work is under way to determine whether this is intra or extracellular.

DISCUSSION.

Local peritoneal factors.

Free fluid in the peritoneum must originally derive from blood capillaries. It must then pass through connective tissue and a serous membrane. At the tissue fluid stage the water-binding properties of the connective tissue along with the lymphatics may act effectively to prevent its further passage into the peritoneal cavity. In these experiments the site of this exchange is apparently the mesenteric, and in addition on transfusion, the retroperitoneal areas. Opie and Rothbard (1953) have shown that rat collagen of different types is a very efficient fluid binder. The swelling of the mentioned areas in these experiments, as a reflection of this, might well act as a retarding influence in the passage of capillary filtrate to peritoneum, long enough to permit its carriage away by the local lymphatics. These tissues must act somewhat as a protein trap in view of the maintained high protein content of the transfusion ascitic fluid despite the fall in plasma protein concentration.

Increased lymph flow will occur behind any vein at increased pressure (Starling, 1894), and this flow may rise enormously above resting levels in the thoracic duct after transfusions (Wasserman and Mayerson, 1952; Korner, Morris and Courtice, 1954) or in the liver lymphatics after partial occlusion of the inferior vena cava in the chest (Bolton and Barnard, 1931; Grindlay, Flock and Bollman, 1948). These latter workers concluded that when the limit of flow in the liver lymphatics was exceeded, the fluid exuded from the liver and so formed ascites. Because of lymphatic and muscle disposition in the gut, lymph flow may be very greatly increased, as after I-V infusions, with such effective-

ness as to reduce local tissue fluid accumulation to a minimum. The results presented here certainly indicate that the mesenteric and retroperitoneal areas are not sites from which gross ascites may be formed in the rat. However, supradiaphragmatic constriction of the inferior vena cava in the rat, as mentioned, does not produce any ascites so that the liver in this animal also seems to be an unlikely source.

These experiments have also indicated that in the balance between production and absorption of ascites, the rat is capable of absorbing such large volumes of protein-fluid that the stress imposed in formation must be very gross in order to achieve even small amounts of free fluid.

Once free, the return of ascites to the circulation is governed by a series of new conditions which involve proximity to the diaphragm (Courtice and Steinbeck, 1951b), adequate function of this muscle (Morris, 1953) and lymphatic return through the diaphragm; in rats that is finally via the thoracic duct (Abdou, Reinhardt and Tarver, 1952). Probably the differences detected in volume absorption after graded doses of injected fluid were attributable to its intraperitoneal distribution. At a certain higher volume of ascites, fluid would come into constant contact with the diaphragm and then be absorbed at a steady rate independent of any further increase in volume.

Distant factors affecting serous effusions.

Portal obstruction has a continued systemic effect on the distribution of body fluid in the rat, for under the stress of I-V infusions in these experiments. ascites formed did not bear a simple relation to the oedema load as it did in normals, where on the average it represented about 1 p.c. of the total load. In susceptible portal occluded rats, transfusion irrespective of load induced a marked ascites. The increase in pleural effusion in the 1-month group may be related to its possible origin in the peritoneum. Lake, Simmonds and Steinbeck (1953) have shown that the pleural fluid which appears after intraperitoneal injections of foreign plasma in rats is fluid absorbed from the peritoneum which has leaked from retrosternal lymphatics. Failure to load the 4-day portal tied group underlines a systemic effect which prevents more prominent ascites formation than does occur on transfusion. This group of rats is subject to an osmotic diuresis in respect of its nitrogen excretion. It is apparent from these results that serous accumulations in the rats are only partly subject to immediate local factors.

SUMMARY.

An analysis of some of the factors likely to affect the presence of experimental ascites has been performed in normal rats and those with partial portal vein occlusion.

Studies of the absorptive capacity of the peritoneal cavity of the rat have

shown that very large volumes of protein-fluid, as much as one-third of the rats' body weight, may be carried away by the lymphatics over 24 hours. This result may be lowered by access to food, especially in the presence of acute portal obstruction.

The rate of absorption of small amounts of ascites is proportional to its volume. With large amounts that are in constant contact with the diaphragm, the maximal rate is independent of further increases in volume.

Massive Ringer-Locke infusions which impose large oedema loads show that the mechanism for return of tissue fluid, along with the large absorption reserve of the peritoneum, is effective in preventing large ascitic accumulations.

Notwithstanding this, after transfusion, 1-month portal tied rats are more likely to form ascites and pleural effusions for an equivalent load than normals. Though on the Starling hypothesis, an earlier 4-day portal vein tied group is more liable to form ascites, this group does not form more than normal. These differences are examined.

The effects of the various procedures on organ weights are presented.

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